

PD-L1 blockade restores CAR T cell activity through IFN γ -regulation of CD163+ macrophages

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29

30 **Short Title: Combining PD-L1 blockade and CAR T cells inhibits macrophage-mediated**
31 **immune suppression**

32 **Keywords:** chimeric antigen receptor, tumor-associated macrophages, M2 macrophages, PD-
33 L1 blockade, immune suppression, tumor microenvironment, prostate cancer, prostate stem cell
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35 Abstract

36 **Background:** The immune suppressive tumor microenvironment (TME) that inhibits T cell
37 infiltration, survival, and anti-tumor activity has posed a major challenge for developing effective
38 immunotherapies for solid tumors. Chimeric antigen receptor (CAR)-engineered T cell therapy
39 has shown unprecedented clinical response in treating patients with hematological
40 malignancies, and intense investigation is underway to achieve similar responses with solid
41 tumors. Immunologically cold tumors, including prostate cancers, are often infiltrated with
42 abundant tumor-associated macrophages (TAMs), and infiltration of CD163 $^{+}$ M2 macrophages
43 correlates with tumor progression and poor responses to immunotherapy. However, the impact
44 of TAMs on CAR T cell activity alone and in combination with TME immunomodulators is
45 unclear.

46 **Methods:** To model this *in vitro*, we utilized a novel co-culture system with tumor cells, CAR T
47 cells, and polarized M1 or M2 macrophages from CD14 $^{+}$ PBMCs collected from healthy human
48 donors. Tumor cell killing, T cell activation and proliferation, and macrophage phenotypes were
49 evaluated by flow cytometry, cytokine production, RNA sequencing, and functional blockade of
50 signaling pathways using antibodies and small molecule inhibitors. We also evaluated the TME
51 in humanized mice following CAR T cell therapy for validation of our *in vitro* findings.

52 **Results:** We observed inhibition of CAR T cell activity with the presence of M2 macrophages,
53 but not M1 macrophages, coinciding with a robust induction of PD-L1 in M2 macrophages. We
54 observed similar PD-L1 expression in TAMs following CAR T cell therapy in the TME of
55 humanized mice. PD-L1, but not PD-1, blockade in combination with CAR T cell therapy altered
56 phenotypes to more M1-like subsets and led to loss of CD163 $^{+}$ M2 macrophages via IFN γ
57 signaling, resulting in improved anti-tumor activity of CAR T cells.

58 **Conclusion:** This study reveals an alternative mechanism by which the combination of CAR T
59 cells and immune checkpoint blockade modulates the immune landscape of solid tumors to
60 enhance therapeutic efficacy of CAR T cells.

61 **Introduction**

62 Adoptive transfer of chimeric antigen receptor (CAR)-engineered T cells has demonstrated
63 robust and durable clinical efficacy in patients with B-cell malignancies,[1-3], but to date has
64 shown underwhelming response rates in patients with solid tumors,[4, 5]. This clinical
65 observation is in large part attributed to the immune-suppressive tumor microenvironment
66 (TME) of solid tumors, comprising infiltrating myeloid cells and regulatory T cells that inhibit
67 endogenous anti-tumor immunity and adoptively transferred cell therapies. Overcoming this
68 challenge will be critical to unleashing the full potential for CAR T cell therapies for solid tumors,
69 and likely will require disease- and context-specific considerations.

70

71 Tumor-associated macrophages (TAMs) are the most abundant immune cells in many solid
72 tumors, and TAM infiltration strongly correlates with tumor progression and poor prognosis in
73 various solid tumors,[6-10] and lymphoma,[11]. While macrophages retain phenotypic and
74 functional plasticity, the majority of TAMs are immune-suppressive, M2-like macrophages with
75 complex pro-tumor functions. TAMs secrete various cytokines and growth factors including IL-
76 10, TGF β , VEGF, and CXCL12 to drive cancer progression through immune suppression, tumor
77 angiogenesis, invasion and metastasis,[12-14]. TAMs also play critical roles in response and
78 resistance to common cancer therapies such as chemotherapy, radiation therapy,[15],
79 angiogenesis,[16] and hormone deprivation therapy,[17], and numerous macrophage-
80 modulating approaches have shown improved therapeutic efficacy in preclinical studies,[12, 18-
81 21].

82

83 Preclinical studies also demonstrated that TAMs mediate resistance to immune checkpoint
84 blockade (ICB),[22-24], and targeting TAMs likely alter outcomes of clinical interventions,[25].
85 PD-1 and PD-L1 are expressed in various immune cells including T cells,[26], NK cells,[27] and
86 macrophages,[28]. Tumor PD-L1 expression does not accurately predict clinical response to

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87 anti-PD-L1 therapy, and more recent studies indicate that PD-L1 expressed by immune cells
88 may contribute to immune suppression,[27-29]. Macrophage PD-L1 is particularly abundant in
89 the TME, but the role of PD-L1 signaling in macrophages and the direct impact of anti-PD-L1
90 blockade on macrophages remains controversial,[28-30]. Recent studies have shown that CAR
91 T cells, especially in combination with other therapeutic agents, modulate myeloid cell
92 phenotypes and alter the immune-suppressive TME,[31-33]. ICB has been utilized in
93 combination with CAR T cell therapy, with the notion that induction of immune responses with
94 CAR T cells may instigate checkpoint pathways in immunologically cold tumors as a
95 compensatory resistance mechanism, providing rationale for the therapeutic combination.
96 Despite a clinical need for overcoming immune suppression to improve CAR T cell therapies for
97 solid tumors, preclinical modeling of this phenomenon is complicated and remain limited in their
98 predictive capabilities.

99

100 In this study, we aimed to develop an *in vitro* model to recapitulate the suppression of CAR T
101 cells in microenvironments with abundant immune-suppressive macrophages. In this model
102 system, target tumor cells and CAR T cells were co-cultured in the presence of M1- or M2-
103 polarized macrophages to evaluate their respective roles in CAR T cell functionality. We showed
104 that M1 macrophages promote, while M2 macrophages suppress, CAR T cell-mediated tumor
105 cell killing and cytokine production. We also observed CAR T cell-regulated PD-L1 induction in
106 both tumor cells and macrophages *in vitro*, with induction levels found to be most dramatic in
107 M2 macrophages. We confirmed CAR T cell-regulated PD-L1 induction in TAMs using an *in vivo*
108 humanized mouse model of prostate cancer. By blocking PD-L1 with atezolizumab or avelumab,
109 we found that inhibiting macrophage PD-L1 was sufficient to restore CAR T cell-mediated tumor
110 killing. However, this restoration of CAR T cell killing by blockade of PD-L1 appears
111 independent of canonical PD-1/PD-L1 signaling, as the phenomenon was not seen with
112 blockade of PD-1 with nivolumab. Instead, PD-L1 inhibition specifically and potently depleted

113 M2 macrophages in the presence of CAR T cells. These findings give mechanistic insights by
114 which CAR T cell and ICB combination therapies enhance anti-tumor immunity in an immune-
115 suppressive TME and is a useful model to study macrophage-mediated immune suppression.

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116 Results

117 Human Monocyte-Derived M2 Macrophages Suppress CAR T Cells *In Vitro*

118 Macrophages are an abundant immune cell population in lymphoma,[11] and various solid
119 tumors including prostate cancer,[7, 8], and their abundance correlates with metastasis and
120 poor prognosis. To investigate the impact of macrophage-rich immunosuppressive solid tumor
121 microenvironment (TME) on CAR T cells, we developed an *in vitro* immune-suppression assay
122 by co-culturing CAR T cells, M1 or M2 macrophages and target tumor cells at an
123 effector:macrophage:tumor ratio of 1:5:10 (**Figure 1a**). Macrophages were differentiated from
124 CD14 $^{+}$ cells enriched from healthy donor PBMCs and *in vitro* polarized as previously
125 described,[34] into M1 (CD80 $^{\text{high}}$, CD163 $^{-}$, CD206 $^{\text{low}}$) or M2 (CD80 $^{\text{low}}$, CD163 $^{+}$, CD206 $^{\text{high}}$)
126 macrophages (**Figure S1a, b**). To model the prostate TME, DU145 prostate tumor cells were
127 engineered to express prostate stem cell antigen (PSCA) and co-cultured with untransduced
128 (UTD) or PSCA-CAR T cells previously developed by our group,[35]. CD19-CAR T cells,[36]
129 and Daudi lymphoma cells were used to model the lymphoma TME. We evaluated antitumor
130 activity, activation and proliferation of CAR T cells by flow cytometry (**Figure S2**) and interferon-
131 γ (IFN γ) secretion by ELISA. CAR T cell anti-tumor activity was normalized to UTD T cells, and
132 activation was measured by 4-1BB upregulation. In both prostate and lymphoma models, anti-
133 tumor cytolytic activity of T cells was inhibited in the presence of M2 macrophages, while it was
134 enhanced in the presence of M1 macrophages (**Figure 1b-d**). T cell proliferation (**Figure 1e**),
135 activation (**Figure 1f, g, Figure S3**), and IFN γ secretion (**Figure 1h**) were also inhibited by M2
136 macrophages. Collectively, these data show that our *in vitro* co-culture system effectively
137 recapitulates the immunosuppressive effects of M2 macrophages on CAR T cells in the TME.

138

139 CAR T Cells Alter the Phenotype of M2 Macrophages *In Vitro*

140 Next, we investigated the impact of CAR T cells on the TME by evaluating phenotypic changes
141 that CAR T cells induce in macrophages. In the immune-suppression assay, we assessed
142 expression of CD80 and CD163 as classical M1 and M2 markers in M2 macrophages in the
143 presence or absence of PSCA-CAR T cells by flow cytometry (**Figure 2a**). We found that CAR T
144 cells upregulated CD80 (**Figure 2b**) and downregulated CD163 (**Figure 2c**) surface expression
145 on M2 macrophages. To evaluate whether such phenotypic changes are mediated by secreted
146 factors, we collected conditioned media from tumor killing assay where DU145-PSCA tumor
147 cells were co-cultured with PSCA-CAR T cells (**Figure 2d**). The conditioned media was applied
148 onto adherent M2 macrophages, and their phenotype was assessed after 48 hours. Phenotypic
149 changes induced in M2 macrophages mirrored the observation in the immune-suppression
150 assay (**Figure 2e, f**), suggesting that CAR T cells alter M2 macrophage phenotype via secreted
151 factors. Furthermore, transcriptome analysis of M2 macrophages by bulk RNA-seq revealed a
152 global gene expression change upon stimulating with the CAR T cell-derived CM, and M1
153 signatures including CD80, CXCL9 and IL1B increased while M2 signatures including CD163,
154 ADORA3 and IL10 decreased (**Figure 2g**). We found by gene ontology analysis that
155 inflammatory pathways were activated (**Figure 2h**), further supporting changes that CAR T cells
156 induce in M2 macrophages. CAR T cell-derived conditioned media did not induce phenotypic
157 changes in M1 macrophages (**Figure S4**). Taken together, these results indicate that CAR T
158 cells alter the TME by repolarizing M2 macrophages to a less immune suppressive, M1-like
159 macrophage state via paracrine signaling.

160

161 **PD-L1 is upregulated in M2 macrophages in the presence of CAR T cells**

162 IFN γ is a well-known inducer of programmed death-ligand 1 (PD-L1) and one of the cytokines T
163 cells secrete upon activation and has been suggested to be a pathway of resistance to cellular
164 immunotherapy,[28, 37]. Therefore, we assessed PD-L1 expression changes in M2
165 macrophages and tumor cells in the immune-suppression assay. In the prostate model, both

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166 DU145-PSCA tumor cells and M2 macrophages induced PD-L1 surface expression in the
167 presence of CAR T cells. Interestingly, M2 macrophages showed greater induction in frequency
168 and abundance of PD-L1 expression compared to tumor cells and M1 macrophages (**Figure 3a-c**). In the lymphoma model, PD-L1 was induced in M2 macrophages (**Figure S5a**) but not in
170 Daudi tumor cells (**Figure S5b**). We hypothesized that PD-L1 was induced in a paracrine
171 fashion, and to test this hypothesis, we treated M2 macrophages and various tumor cells with
172 conditioned media obtained from tumor killing assays. PD-L1 induction was the greatest in M2
173 macrophages at the protein (**Figure S6a, b**) and mRNA levels (**Figure S6c**), recapitulating
174 induction in the *in vitro* immune suppression assay.

175

176 To evaluate whether CAR T cells induce PD-L1 expression in tumor-associated macrophages *in*
177 *vivo*, we humanized mice by engrafting human CD34 $^{+}$ hematopoietic stem cells in immune-
178 compromised MISTRG mice,[38]. DU145-PSCA tumor cells were then injected subcutaneously,
179 and LAPC9 cells, a patient-derived metastatic prostate cancer cell line with endogenous PSCA
180 expression, were injected into the intratibial space to model bone metastatic disease,[35].
181 PSCA-CAR T cells were adoptively transferred via intravenous injection, as we have done
182 previously in our preclinical therapeutic studies,[35]. In humanized MISTRG mice, CD68 $^{+}$
183 human macrophages efficiently infiltrated into human tumor xenografts, and immunostaining
184 revealed colocalization of CD68 and PD-L1 in DU145-PSCA (**Figure 3d**) and in LAPC9 (**Figure**
185 **3e**) xenograft. These data, collectively, show that CAR T cells directly induce PD-L1 in both
186 tumor cells and M2 macrophages *in vitro* and *in vivo*.

187

188 **IFN γ is not a dominant inducer of PD-L1 expression by CAR T cells**

189 We next hypothesized that PD-L1 is induced by IFN γ , and to test the hypothesis, we treated M2
190 macrophages and DU145 tumor cells with conditioned media collected from the tumor killing

191 assay in the presence of anti-IFN γ R1 antibody. Cells were collected after 48 hours to evaluate
192 PD-L1 protein expression by flow cytometry (**Figure 3f, Figure S7a**), and cell lysates were
193 collected after 6 hours to measure mRNA expression by qPCR (**Figure 3g, Figure S7b**).
194 Blocking IFN γ signaling was not sufficient to inhibit PD-L1 expression in M2 macrophages or
195 DU145 tumor cells in the conditioned media. Also, recombinant IFN γ only moderately induced
196 PD-L1 expression when it was added at similar concentrations (~20 ng/ml) measured in CAR T
197 cell-derived conditioned media (**Figure S8a**). Increasing the concentration of recombinant IFN γ
198 up to 200 ng/ml did not reach the level of PD-L1 induction in M1 and M2 macrophages observed
199 with CAR T cell-conditioned media (**Figure S8b, c**). We treated M1 and M2 macrophages with
200 varying concentrations of conditioned media and showed that 5-20% conditioned media was
201 sufficient to induce maximal levels of PD-L1 (**Figure S8d**). Despite IFN γ being a well-
202 established PD-L1 inducer, these results indicate that IFN γ is not a sole or dominant inducer of
203 PD-L1 expression in tumor cells or M2 macrophages in this system. The data suggest that PD-
204 L1 induction is regulated by the presence of other inducers in CAR T cell-derived soluble
205 factors.

206
207 To identify signaling pathways that mediate PD-L1 induction, we treated M2 macrophages with
208 small molecule inhibitors of various pathways. While inhibition of STAT3, NF κ B, AKT, PI3K and
209 mTOR signaling was not sufficient to block PD-L1 induction by CAR T cells in M2 macrophages,
210 inhibition of STAT1 with fludarabine resulted in loss of PD-L1 induction in M2 macrophages
211 (**Figure 3h**). Loss of PD-L1 induction was also shown following JAK1/2 inhibition with AZD1480
212 as well as JAK1-selective inhibition with itacitinib, but not by JAK2 inhibition with AG490. These
213 results indicate that PD-L1 expression induced by CAR T cells is mediated primarily by a
214 JAK1/STAT1 pathway, independent of IFN γ .

215

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216 PD-L1 blockade inhibits M2 macrophage-mediated suppression of CAR T cells

217 To test the functionality of PD-L1 in immune suppression by M2 macrophages, we blocked PD-
218 L1 with atezolizumab, an anti-PD-L1 monoclonal antibody, in the *in vitro* immune suppression
219 assay. PD-L1 blockade restored CAR T cell-mediated tumor cell killing in the presence of M2
220 macrophages (**Figure 4a, b**). This observation was reproduced using avelumab, another anti-
221 PD-L1 monoclonal antibody (**Figure 4e**). T cell activation (**Figure 4c**) and IFN γ secretion
222 (**Figure 4d**) were also restored in the presence of PD-L1 blockade, supporting a role for PD-L1
223 in regulating M2 macrophage-mediated immune suppression. Furthermore, tumor killing was
224 also restored in the lymphoma model (**Figure S9**) where Daudi tumor cells lacked PD-L1
225 expression (**Figure S5b**). These data indicate that macrophage PD-L1 is sufficient to drive
226 immune suppression. However, blocking PD-1 using Nivolumab, an anti-PD-1 monoclonal
227 antibody, did not restore CAR T cell-mediated tumor cell killing in a similar fashion (**Figure**
228 **S10a**). Interestingly, consistent with our previous publication,[35], PD-1 was not readily induced
229 in CAR T cells (**Figure S10b**). Therefore, while M2 macrophage PD-L1 is necessary for immune
230 suppression in this system, these results indicated that the classical PD-1/PD-L1 signaling axis
231 is not a primary mechanism by which M2 macrophages suppress CAR T cells.

232

233 Combining CAR T cells and PD-L1 blockade alter phenotype and reduce survival of M2 234 macrophages

235 Macrophages express PD-1 and PD-L1 (**Figure S1b**), and increasing evidence supports that
236 these cell surface receptors play a role in shaping intrinsic cellular properties of macrophages
237 including their immune suppressive function,[30, 39, 40]. We hypothesized that blocking PD-L1
238 alters the ability of M2 macrophages to suppress CAR T cells. First, we assessed M2
239 macrophages in the immune-suppression assay in the presence of PD-L1 blockade. In the
240 presence of CAR T cells, the number of M2 macrophages decreased compared to respective
241 controls with UTD T cells (**Figure 4a, Figure 5a**). The combination of CAR T cells and PD-L1

242 blockade resulted in significantly fewer M2 macrophages, and specifically in the presence of
243 CAR T cells. These data suggest that PD-L1 blockade specifically in combination with CAR T
244 cells has a direct impact on survival of M2 macrophages. We also evaluated macrophage
245 phenotype and found fewer CD163⁺ M2 macrophages in the combination of CAR T cells and
246 PD-L1 blockade (**Figure 5b**). To further interrogate the mechanism underlying this
247 phenomenon, we stimulated M2 macrophages with conditioned media collected from tumor-
248 CAR T cell co-cultures. Consistent with the previous observation in the immune suppression
249 assay, the combination of CAR T cells and PD-L1 blockade resulted in reduction of total viable
250 and CD163+ M2 macrophages (**Figure 5c, d**). Given previous studies in the field suggesting the
251 importance of PD-1 in immune suppression by macrophages,[39, 40], we also blocked PD-1
252 with Nivolumab, but did not observe a similar impact on viability or changes in CD163
253 expression in M2 macrophage (**Figure 5c, d**).

254

255 **IFN γ signaling mediates altered phenotype of M2 macrophages following PD-L1 inhibition**
256 IFN γ activates macrophages and plays important roles in promoting inflammation. Therefore, we
257 hypothesized that IFN γ mediates the loss of CD163⁺ M2 macrophages in the combination of
258 CAR T cells and PD-L1 blockade. To test this, we treated M2 macrophages with anti-PD-L1 and
259 anti-IFN γ R1 antibodies in the conditioned media collected from tumor-CAR T cell co-cultures. By
260 microscopy, we not only visually confirmed the reduction in M2 macrophage cell numbers with
261 PD-L1 inhibition, but also observed M2 macrophages become enlarged and vacuolated (**Figure**
262 **5e**). Blocking IFN γ signaling prevented these morphological changes and loss of CD163⁺ cells
263 induced by PD-L1 inhibition (**Figure 5e, f, g**), suggesting that the combination of CAR T cells
264 and PD-L1 blockade directly impacts M2 macrophages via IFN γ signaling, reversing M2
265 macrophage-mediated immunosuppression of CAR T cells.

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266 Discussion

267 In the current study, we investigated the impact of myeloid cells on CAR T cell activity using an
268 *in vitro* model that we established to recapitulate the immune-suppressive TME. We found that
269 M2 macrophages, but not M1 macrophages, suppressed the anti-tumor activity of CAR T cells
270 using both PSCA $^{+}$ prostate cancer and CD19 $^{+}$ lymphoma models. The presence of CAR T cells
271 altered the phenotype of M2 macrophages towards a less immune-suppressive state with
272 reduced M2-like CD163 $^{+}$ and greater M1-like CD80 $^{+}$ populations. We also observed induction of
273 PD-L1 expression in tumor cells as well as M1 and M2 macrophages, but M2 macrophages had
274 significantly higher cell-surface density of PD-L1 induction than in tumor cells or M1
275 macrophages. Inhibition of PD-L1 using antibody blockade restored CAR T cell function
276 suppressed by M2 macrophages, but this restoration was not mediated by canonical PD-1/PD-
277 L1 axis as CAR T cell function was not restored with PD-1 blockade. Instead, the combination of
278 CAR T cells and PD-L1 blockade resulted in fewer CD163 $^{+}$ M2 macrophages, suggesting a
279 direct impact on these cells. Further, we showed that IFN γ was required for this phenomenon,
280 as inhibition of IFN γ R signaling potently reversed this PD-L1-regulated survival of M2
281 macrophage. These findings provide mechanistic insight into CAR T cell-mediated alterations in
282 the TME and specifically on immune-suppressive myeloid cells. However, our studies suggest
283 CAR T cells alone may not be sufficient to overcome immunosuppression in the TME and may
284 require PD-L1 blockade to enable the full therapeutic potential of CAR T cells.

285

286 While recent evidence supports the notion that CAR T cells alone can enhance endogenous
287 immunity, numerous studies have shown that CAR T cell therapy is not able to elicit adequate
288 clinical response against solid tumors,[41, 42], justifying rational for combining
289 immunotherapies. Our *in vitro* model confirms the ability of CAR T cells to alter the myeloid cell
290 subsets to a less suppressive state, but such immunomodulation was not sufficient for CAR T

291 cells to evade immune suppression. Moreover, we observed this M2 macrophage shift to a
292 more pro-inflammatory state in approximately 60% of tested healthy human donors,
293 demonstrating apparent heterogeneity in CAR T cell-mediated immunomodulation and
294 susceptibility of macrophages among individuals. Studies in mouse models might reproduce
295 immunomodulation of macrophages in response to CAR T cells, but the use of inbred mice
296 might not adequately uncover heterogenous responses that we observed in our *in vitro* model.
297 We may be able to use this model in the future to better understand and develop therapies that
298 enhance how CAR T cells function in the presence of TMEs with abundant M2 macrophage
299 subsets as seen in prostate cancers and other solid tumors.

300

301 PD-1/PD-L1 blockade combined with CAR T cells is a current clinical strategy owing largely to
302 the field's collective evidence that immune checkpoint pathways are induced following activity of
303 CAR T cells, which may ultimately lead to exhaustion of CAR T cells. The contribution of
304 myeloid PD-L1 expression on immunosuppression within the tumor microenvironment has been
305 reported in preclinical models and could be regulated by alternative mechanisms ,[28, 30]. In
306 our study, the canonical PD-1/PD-L1 axis did not directly influence CAR T cell function, as PD-
307 L1 blockade, but not PD-1 blockade, reversed macrophage-mediated immune suppression. Our
308 data suggest that CAR T cell-mediated PD-L1 expression in macrophages may specifically and
309 directly drive their survival and immune-suppressive phenotype. The change in CD163
310 expression of macrophages in response to CAR T cells was variable among individuals,
311 however, combining CAR T cells with PD-L1 blockade induced a uniform response in all tested
312 individuals. Loss of immune-suppressive macrophages with the combination of CAR T cells and
313 PD-L1 blockade resembles observations with other myeloid-targeting therapies, including
314 CSF1/CSF1R blockade^{14,36}, CCL2/CCR2 inhibition,[13, 20] and novel anti-CD206 peptides,[18].
315 Due to this mechanism of action of TME remodeling, the efficacy of combining CAR T cells and
316 PD-L1 blockade may be driven in part by tumor composition and density of macrophages. Our

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317 data suggest that this combination therapy may be more effective in immunologically “cold” solid
318 tumors with abundant CD163 $^{+}$ immune suppressive macrophages.

319

320 The requirement of IFN γ in regulating the survival and function of M2 macrophage following PD-
321 L1 blockade suggests that amplifying IFN γ signaling may be an actionable target for improving
322 the combination of CAR T cells and ICB. Various engineering and manufacturing approaches
323 can enhance IFN γ secretion by CAR T cells,[35, 36, 43, 44], and CAR T cells with greater IFN γ
324 secretion may better remodel the TME in combination with ICB. Although we found that IFN γ
325 was critical for PD-L1 blockade-induced M2 macrophage depletion, mechanisms of how the
326 combination impact functions of immune suppressive macrophages remains unclear. Although
327 increased apoptosis of CD163 $^{+}$ cells in the combination of CAR T cells and PD-L1 blockade was
328 expected, we failed to demonstrate increased apoptosis in our studies. Using time-lapsed
329 imaging, we revealed cells pursuing and catching adjacent cells before morphological changes
330 occurred, indicating possible antibody-dependent cellular phagocytosis of M2 macrophages.
331 Also, macrophages are known to enlarge and form vacuoles via fusion in chronic
332 inflammation,[45], and the morphological changes may be a manifestation of a highly
333 inflammatory state. Further studies are warranted to elucidate mechanisms of
334 immunomodulation that macrophages undergo following CAR T cell therapy and PD-L1
335 blockade.

336

337 We built the immune suppression assay under an assumption that TAMs are M2-like, immune
338 suppressive macrophages. However, macrophages phenotypes and functions are not as binary
339 as M1 or M2, but rather demonstrate plasticity along a spectrum of phenotypes and functions. In
340 addition to macrophage cell plasticity, the disease context and clinical interventions likely
341 contribute to shaping the phenotype of TAMs. It is difficult to predict this spectrum of

342 macrophage phenotypes using our *in vitro* system. However, our study addresses potential
343 mechanisms underlying CAR T cell and PD-L1 blockade alone and in combination. While our
344 studies did not include validation of this combination therapy approach using *in vivo* models, our
345 histological evaluation of tumors in humanized MISTRG mice do confirm increased PD-L1
346 expression in TAMs following CAR T cell therapy. We previously developed and published an
347 immunocompetent mouse model where we assessed safety and efficacy of PSCA-CAR T cells
348 in murine cancers,[33]. Future studies will evaluate the combination using this syngeneic mouse
349 model. Additionally, future clinical trials to evaluate safety and efficacy of combining CAR T cell
350 therapy and ICB in solid cancers and lymphoma may corroborate our findings.

351

352 To our knowledge, this is the first example of a mode of action of ICB by which myeloid cells are
353 directly targeted and depleted specifically in the context of CAR T cell therapy, and this study
354 gives new insights to a mechanism by which PD-L1-negative tumors may benefit from CAR T
355 cell therapy in combination specifically with PD-L1 blockade. The altered phenotypes and
356 depletion of immune-suppressive macrophages in tumors may require both CAR T cells and
357 PD-L1 blockade and warrants further engineering of CAR T cells to secrete PD-L1 blockers and
358 enhance IFN γ signaling to improve anti-tumor responses in TAM-rich solid tumors.

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359 **Materials and Methods**

360 **Cell lines**

361 Human metastatic prostate cancer cell lines DU145 (ATCC HTB-81) and PC-3 (ATCC CRL-
362 1435), human lymphoma cell line Daudi (ATCC CCL-213), and human monocytic leukemia cell
363 line THP-1 (ATCC TIB-202) were cultured in RPMI-1640 (Lonza, 12-115F) containing 10% fetal
364 bovine serum (FBS, Hyclone, SH30070.03) (RPMI+10%FBS). DU145 and PC-3 tumor cells
365 were engineered to express PSCA antigen as previously described,[35]. Human pancreatic
366 cancer cell line HPAC (ATCC CRL-2119) and human breast cancer cell line MDA-MB-231
367 (ATCC CRM-HTB-26) were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-
368 12 (DMEM/F12, Corning, 10-092-CV) containing 10% FBS. MCF-7 (ATCC HTB-22) breast
369 cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, 11960-051)
370 containing 10% FBS, 25 mM HEPES (Irvine Scientific, 9319), and 2mM L-Glutamine (Lonza,
371 17-605E). Patient-derived metastatic prostate cancer LAPC-9 cells used *in vivo* were
372 generously provided by the Reiter Lab at UCLA. LAPC9 cells were engineered to express
373 eGFP/firefly luciferase (LAPC-9-eGFP-ffLuc) and maintained as described in previous
374 literature.[35].

375

376 **DNA Construction and Lentivirus production**

377 PSCA- and CD19-targeting CARs were designed as previously described, and respective
378 constructs carried truncated CD19 and EGFR as a surrogate marker of transduction,[35, 36].
379 Lentivirus was manufactured following previously established methods,[35]. In short, lentivirus
380 was generated using 293T cells in T-225 flasks and cultured overnight prior to transfection with
381 packaging plasmids and desired lentiviral backbone plasmid. Supernatants containing lentivirus
382 were collected following 3 to 4 days, filtered, and centrifuged to remove residual cell debris.

383 Lentivirus containing supernatant then underwent incubation with 2mM magnesium and 25U/mL
384 Benzonase endonuclease. Suspended lentivirus was then concentrated by high-speed
385 centrifugation (6080 x g) overnight at 4°C. Lentiviral pellets were resuspended in PBS-lactose
386 solution (4g lactose per 100mL PBS) then aliquoted and stored at -80°C until ready for use.
387 Lentiviral titers were determined using Jurkat cells.

388

389 **PBMC and monocyte isolation**

390 Leukapheresis products were obtained from consented research participants (healthy donors)
391 under protocols approved by the City of Hope Internal Review Board (IRB). On the day of
392 leukapheresis, peripheral blood mononuclear cells (PBMC) were isolated by density gradient
393 centrifugation over Ficoll-Paque (GE Healthcare) followed by multiple washes in PBS/EDTA
394 (Miltenyi Biotec).

395

396 Monocytes were isolated from freshly collected PBMCs using CD14 antibody-conjugated
397 microbeads and magnetic columns (Miltenyi Biotec) according to the manufacturer's protocol.
398 CD14⁺ monocytes and CD14⁻ fraction were frozen in CryoStor[®] CS5 (StemCell Technologies)
399 until processed further.

400

401 **T cell lentiviral transduction and ex vivo expansion**

402 T cell activation and transduction was performed as described previously,[35]. Briefly, freshly
403 thawed CD14⁻ or whole PBMCs were washed once and cultured in X-VIVO-15 (Lonza) with
404 10% FBS (complete X-VIVO) containing 100 U/mL recombinant human IL-2 (rhIL-2, Novartis
405 Oncology) and 0.5 ng/mL recombinant human IL-15 (rhIL-15, CellGenix). For CAR lentiviral
406 transduction, T cells were cultured with CD3/CD28 Dynabeads[®] (Life Technologies), protamine

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407 sulfate (APP Pharmaceuticals), cytokine mixture (as stated above), and desired lentivirus at a
408 0.1-1 multiplicity of infection (MOI) the day following stimulation. Cells were then cultured in and
409 replenished with fresh complete X-VIVO containing cytokines every 2–3 days. After 7 days,
410 beads were magnetically removed, and cells were further expanded in complete X-VIVO
411 containing cytokines to achieve desired cell yield. CAR T cells were positively selected for
412 truncated CD19 using the EasySepTM CD19 Positive Enrichment Kit I or II (StemCell
413 Technologies) (for PSCA-CAR T cells) or positively selected for truncated EGFR using a custom
414 EasySepTM EGFR Positive Enrichment Kit (for CD19-CAR T cells) according to the
415 manufacturer's protocol. Following further expansion, cells were frozen in CryoStor[®] CS5 prior
416 to *in vitro* functional assays and *in vivo* therapeutic models. Purity and phenotype of CAR T cells
417 were verified by flow cytometry.

418

419 ***In vitro* macrophage differentiation**

420 Primary human M1 and M2 macrophages were differentiated and polarized as previously
421 described,[34]. Briefly, frozen human monocytes were thawed and cultured in cytokine-
422 containing RPMI+10% FBS for 7-10 days. To differentiate M1 macrophages, cells were cultured
423 with GM-CSF (BioLegend, 572903). The media was changed once after 3-5 days to media
424 containing GM-CSF, IFN γ (BioLegend, 570202), LPS (Sigma-Aldrich, L3012-5MG) and IL-6
425 (BioLegend, 570804). To differentiate M2 macrophages, cells were cultured with M-CSF
426 (BioLegend, Cat: 574804). The media was changed once after 3-5 days to media containing M-
427 CSF, IL-4 (BioLegend, 574004), IL-13 (BioLegend, 571102) and IL-6. All cytokines and LPS
428 were used at 20ng/mL. After differentiation, macrophages were lifted using PBS + 1mM EDTA
429 (PBS-EDTA, Cellgro), and phenotype was assessed by flow cytometry to confirm successful
430 polarization. Cells were counted and used for further studies.

431

432 To differentiate and polarize M1 and M2 macrophages from human monocytes THP-1 (ATCC
433 TIB-202), commonly used protocols were adapted,[46, 47]. THP-1 cells were stimulated with
434 phorbol 12-myristate 13-acetate (PMA) for 24 hours and rested for 72 hours in RPMI+10% FBS.
435 Cells were then polarized for 24 hours to M1 or M2 macrophages in the presence of IFN γ and
436 LPS or IL-4 and IL-13, respectively. Cytokines and PMA were used at 20ng/mL. Polarized
437 macrophages were lifted with PBS-EDTA and used for further studies.

438

439 **Flow cytometry**

440 For flow cytometric analysis, cells were resuspended in FACS buffer (Hank's balanced salt
441 solution without Ca²⁺, Mg²⁺, or phenol red (HBSS^{-/-}, Life Technologies) containing 2% FBS and
442 0.5% Sodium Azide. Cells were incubated with primary antibodies for 30 min at 4°C in the
443 dark. Cell viability was determined using 4',6-diamidino-2-phenylindole (DAPI, Sigma). Flow
444 cytometry was performed on a MACSQuant Analyzer 10 (Miltenyi Biotec), and the data were
445 analyzed with FlowJo software (v10, TreeStar). Antibodies targeting human CD45 (BD
446 Biosciences, 347464), CD137 (BD Biosciences, 555956), CD19 (BD PharmingenTM, 557835),
447 EGFR (BioLegend, 352906), CD80 (BD Biosciences, 340294), CD163 (eBioscience, 17-1639-
448 42), CD206 (BioLegend, 321123), PD-L1 (BD Biosciences, 558065), PD-1 (eBioscience, 47-
449 2799-42), CD33 (BD Biosciences, 340533), HLA-DR (eBioscience, 47-9956-42), and CSF1R
450 (BioLegend 347305) were used for analysis.

451

452 **ELISA**

453 IFN γ in supernatant was measured using Human IFN γ ELISA Kit (Invitrogen, 88-7316-88)
454 according to the manufacturer's protocol. Plates were read at 450 nm using Cytation 5 (BioTek).

455

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456 ***In vitro* immune-suppression assay**

457 CAR T cells, macrophages, and target tumors were co-cultured in RPMI+10% FBS in the
458 absence of exogenous cytokines in 96-well plates. Cells were plated at an
459 effector:macrophage:target (E:M:T) ratio of 1:5:10 to model prostate cancer with DU145-PSCA
460 cells and lymphoma with Daudi cells. For analysis of the prostate cancer model, supernatant
461 was collected after 3 days for ELISA, and cells were trypsinized and collected for flow cytometry
462 after 6 or 10 days. T cell proliferation was assessed after 10 days, and all other parameters
463 including tumor cell killing, T cell activation and macrophage phenotype were evaluated after 6
464 days by flow cytometry. The lymphoma model was analyzed after 3 days of culture.

465

466 **Generation of CAR T cell-derived conditioned media**

467 PSCA-CAR T cells or untransduced (UTD) controls (5×10^3) were co-cultured with DU145-
468 PSCA cells (5×10^4) for 72 hours, and supernatant was collected and centrifuged at 500 x g for
469 5 minutes. Cell-free conditioned media (CM) was collected and stored at -80°C. CAR T cell
470 function was validated by flow cytometry, and when it is mentioned, ELISA was performed prior
471 to using the supernatant to determine concentrations of IFN γ .

472

473 **Stimulation of macrophages with CAR T cell-derived conditioned media**

474 Differentiated macrophages were plated in RPMI+10% FBS and rested overnight, and CAR or
475 UTD T cell-derived CM collected from tumor cell-T cell co-cultures was applied to stimulate
476 macrophages. Cells were analyzed by flow cytometry after 48 hours. Cell morphology was
477 captured by using BZ-X810 Inverted Microscope (Keyence) or Axio Vert.A1 Inverted Microscope
478 (Zeiss). Atezolizumab (anti-human PD-L1, Tecentriq®, Genentech), Avelumab (anti-human PD-
479 L1, Bavencio®, EMD Serono), Nivolumab (anti-human PD-1, Opdivo®, Bristol Meyers Squibb),

480 and isotype control (bgal-mab12, InvivoGen) were added at the time of stimulation. Anti-IFNyR α
481 (BioLegend, 308610) and isotype control (BioLegend, 400166) were added to culture 2 hours
482 prior to stimulation with CM. Similarly, cells were pre-incubated with small molecule inhibitors for
483 30 minutes prior to stimulation. Small molecule inhibitors included Fludarabine (STAT1 inhibitor,
484 EnzoALX-480-100-M005), AZD1480 (JAK1 & JAK2 inhibitor, MilliporeSigma, SML1505-5MG),
485 Itacitinib (JAK1 inhibitor, Cayman Chemicals, 27597), Rapamycin (mTOR inhibitor, Cayman
486 Chemicals, 13346), C188-9 (STAT3 inhibitor, Cayman Chemicals, 30928), Akt Inhibitor VIII
487 (AKT inhibitor, MilliporeSigma, 124018-5MG), BAY 11-7082 (NF- κ B inhibitor, MilliporeSigma,
488 B5556-10MG), AG490 (JAK2 inhibitor, MilliporeSigma, 658401-5MG), C2ZC24832 (PI3K γ
489 inhibitor, MilliporeSigma, SML1214-5MG).

490

491 **RT-PCR**

492 RNA was isolated using RNeasy mini kit (Qiagen) or Quick-RNA Microprep Kit (Zymo
493 Research), and RNA concentration was measured using NanoDrop (Thermo Scientific). cDNA
494 was prepared from 0.4-1 μ g of total RNA using SuperScript IV reverse transcriptase
495 (ThermoFisher Scientific). Quantitative PCR was performed using SsoAdvanced Universal
496 SYBR Green Supermix (Bio-Rad) on CFX96 Real-Time PCR Detection System (Bio-Rad). The
497 data were analyzed by the comparative threshold method, and gene expression was normalized
498 to GAPDH. The following primers were used: CD274: forward, GCTGAACGCCCATACAACA;
499 reverse, TCCAGATGACTTCGGCCTTG and GAPDH: forward, TCGGAGTCAACGGATTGGT;
500 reverse, TTCCCGTTCTCAGCCTTGAC. These primer sets were validated to have a single
501 melting curve and amplification efficiency of 2.

502

503 **RNA sequencing**

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504 Macrophages were stimulated with CAR or UTD T cell-derived CM collected from tumor cell-T
505 cell co-cultures. After 8 hours, cells were lysed using RNA Lysis Buffer (Zymo Research,
506 R1060-1-50), and RNA was isolated according to the manufacturer's protocol. Libraries for
507 stranded poly(A) RNA-seq were created using the KAPA mRNA HyperPrep kit (Roche).

508

509 Sequencing of 51 bp single-end reads was performed using a HiSeq2500 regular run. Base
510 calling (de-multiplexing samples between and within labs by 6 bp barcodes, from a 7 bp index
511 read) was performed using bcl2fastq v2.18. Reads were aligned against the human genome
512 using TopHat2,[48]. Read counts were tabulated using htseq-count,,[49] with UCSC known
513 gene annotations,[50]. Change values were calculated from fragments per kilobase per million
514 (FPKM) reads normalized expression values, which were also used for visualization (following a
515 log2 transformation),[51]. Aligned reads were counted using GenomicRanges,[52]. GSEA was
516 run on log2(FPKM + 0.1) expression values, with upregulated enrichment results for GO
517 Biological Process categories in MSigDB,[53-55].

518

519 **Animal Experiments**

520 All animal experiments were performed under protocols approved by the City of Hope Animal
521 Care and Use Committee (IACUC). MISTRG mice were obtained through MTA from Regeneron
522 Pharmaceuticals and housed and bred at City of Hope. 3-6 week old MISTRG mice were
523 sublethally irradiated (100cGy, J.L. Shepherd Mark I Cs-137 irradiator) 6-12 hours prior to
524 engraftment of human adult G-CSF mobilized CD34 $^{+}$ cells (2.5×10^5) via intravenous injection.
525 Human adult G-CSF mobilized CD34 $^{+}$ cells and autologous PBMCs were purchased from
526 HemaCare, and autologous PBMCs were used to manufacture CAR and UTD T cells used for
527 adoptive cell transfer (ACT). DU145-PSCA cells ($2.5-5 \times 10^5$) were engrafted subcutaneously

528 (s.c.), and tumor growth was monitored by biweekly caliper measurement. For an orthotopic
529 intratibial model, LAPC-9-eGFP-ffLuc cells (1.5×10^5) were engrafted into the intratibial space
530 (i.ti.), and tumor growth was monitored by biweekly non-invasive bioluminescence imaging
531 (Lago-X, Accela). For non-invasive flux imaging, mice were injected intraperitoneally with 150 mL
532 D-luciferin potassium salt (Perkin Elmer) suspended in PBS at 4.29 mg/mouse. Flux signals
533 were analyzed with Aura imaging software (Spectral Instruments Imaging). Mice received ACT
534 of CAR or UTD T cells (1×10^6) when DU145-PSCA s.c. reach $\sim 150\text{mm}^3$ or 14 days after
535 LAPC-9-eGFP-ffLuc i.ti. engraftment. Tumors were harvested 7 days following ACT for
536 histology.

537

538 **Immunohistochemistry and Immunofluorescent Staining**

539 Collected mouse tissue was fixed in 4% paraformaldehyde (4% PFA, Boston BioProducts) and
540 stored in 70% ethanol until processed further. Tissue embedding, sectioning, H&E and IHC
541 staining were performed by the Research Pathology Core at City of Hope.

542

543 Immunofluorescent staining of tissue was completed on paraffin embedded tissue. In brief,
544 paraffin sections were deparaffinized and rehydrated, and antigens were retrieved in citrate-
545 based antigen unmasking solution (Vector Lab, H-3300-250) for 10 minutes at 120°C using an
546 autoclave. Samples were rehydrated, permeabilized with 0.1% Triton X-100 for 30 minutes at
547 room temperature and blocked with 5% normal donkey serum (NDS) for 45 minutes prior to
548 immunostaining. Tissue was incubated with rabbit anti-human CD68 (1:200, Cell Signaling
549 Technology, 76437T) and goat anti-human PD-L1 (1:50, Leinco Technologies, B560) at 4°C
550 overnight and washed in PBS+0.1% Tween 20 for 5 min three times. Tissue was incubated with
551 secondary antibodies donkey anti-rabbit IgG, AlexaFluor 488 (1:1000, Invitrogen, A-21206) and

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552 donkey anti-goat IgG, AlexaFluor 546 (1:1000, Invitrogen, A-11056) for 1 hour at room
553 temperature, washed in PBS+0.1% Tween 20 for 5 min three times and mounted with mounting
554 media containing DAPI (Vector Laboratories). Fluorescent images were captured using BZ-
555 X810 Inverted Microscope (Keyence).

556

557 Double IHC was performed by the Research Pathology Core at City of Hope. Staining was
558 performed on Ventana Discovery Ultra (Ventana Medical Systems, Roche Diagnostics,
559 Indianapolis, USA) IHC Auto Stainer, and mouse anti-human CD68 (Dako, M087601-2) and
560 rabbit anti-human PD-L1 (Ventana, 790-4905) were used at 1:100. Briefly, the slides were
561 loaded on the machine, deparaffinization, rehydration, endogenous peroxidase activity inhibition
562 and antigen retrieval were first performed. Two antigens were sequentially detected and heat
563 inactivation was used between the two antigen detection steps to prevent any potential cross-
564 reactivities. Following the first primary antibody (PD-L1) incubation, DISCOVERY anti-Rabbit
565 NP and DISCOVERY anti-NP-AP were incubated, and stains were visualized with DISCOVERY
566 Yellow Kit. Following the heat inactivation, the second primary antibody (CD68) was incubated,
567 DISCOVERY anti-Rabbit HQ and DISCOVERY anti-HQ-HRP were added, and stains were
568 visualized by DISCOVERY Teal Kit. The slides were then counterstained with hematoxylin
569 (Ventana) and coverslipped. Slides were scanned by using NanoZoomer 2.0HT (Hamamatsu).

570

571 **Statistical Analysis**

572 Data are presented as mean \pm standard error of the mean (SEM) unless otherwise stated.
573 Statistical comparisons between groups were performed using the unpaired two-tailed Student's
574 *t* test to calculate *p* values, unless otherwise stated.

575

576 **Supplemental Information:** Supplemental figures and legends are available and included as
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578

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595 S.J.P, S.J.F., T.B.D., X.W., J.C.Z., V.D.J., N.L., R.N., K.O., J.G., and Y.Y. provided design of
596 experimental procedures, data analysis, and interpretation. Y.Y., J.G., and K.O., performed
597 experiments. Y.Y. and S.J.P. wrote the manuscript. C.M. and J.G. assisted in writing the
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602 **References**

603 1. June CH, O'Connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell
604 immunotherapy for human cancer. *Science (New York, NY)*. 2018;359(6382):1361-5.

605 2. Mirzaei HR, Rodriguez A, Shepphard J, Brown CE, Badie B. Chimeric Antigen Receptors
606 T Cell Therapy in Solid Tumor: Challenges and Clinical Applications. *Frontiers in immunology*.
607 2017;8:1850.

608 3. Neelapu SS, Locke FL, Bartlett NL, Lekakis LJ, Miklos DB, Jacobson CA, et al.
609 Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. *N Engl J
610 Med.* 2017;377(26):2531-44.

611 4. Adusumilli PS, Zauderer MG, Rivière I, Solomon SB, Rusch VW, O'Cearbhaill RE, et al.
612 A Phase I Trial of Regional Mesothelin-Targeted CAR T-cell Therapy in Patients with Malignant
613 Pleural Disease, in Combination with the Anti-PD-1 Agent Pembrolizumab. *Cancer discovery*.
614 2021;11(11):2748-63.

615 5. Ahmed N, Brawley V, Hegde M, Bielamowicz K, Kalra M, Landi D, et al. HER2-Specific
616 Chimeric Antigen Receptor-Modified Virus-Specific T Cells for Progressive Glioblastoma: A
617 Phase 1 Dose-Escalation Trial. *JAMA Oncol.* 2017;3(8):1094-101.

618 6. Nonomura N, Takayama H, Nakayama M, Nakai Y, Kawashima A, Mukai M, et al.
619 Infiltration of tumour-associated macrophages in prostate biopsy specimens is predictive of
620 disease progression after hormonal therapy for prostate cancer. *BJU international*.
621 2011;107(12):1918-22.

622 7. Erlandsson A, Carlsson J, Lundholm M, Fält A, Andersson SO, Andrén O, et al. M2
623 macrophages and regulatory T cells in lethal prostate cancer. *The Prostate*. 2019;79(4):363-9.

624 8. Zarif JC, Baena-Del Valle JA, Hicks JL, Heaphy CM, Vidal I, Luo J, et al. Mannose
625 Receptor-positive Macrophage Infiltration Correlates with Prostate Cancer Onset and Metastatic
626 Castration-resistant Disease. *Eur Urol Oncol*. 2019;2(4):429-36.

627 9. Zhang R, Liu Q, Peng J, Wang M, Gao X, Liao Q, et al. Pancreatic cancer-educated
628 macrophages protect cancer cells from complement-dependent cytotoxicity by up-regulation of
629 CD59. *Cell Death Dis.* 2019;10(11):836.

630 10. Larionova I, Tuguzbaeva G, Ponomaryova A, Stakheyeva M, Cherdynseva N, Pavlov V,
631 et al. Tumor-Associated Macrophages in Human Breast, Colorectal, Lung, Ovarian and Prostate
632 Cancers. *Front Oncol.* 2020;10:566511.

633 11. Shen L, Li H, Shi Y, Wang D, Gong J, Xun J, et al. M2 tumour-associated macrophages
634 contribute to tumour progression via legumain remodelling the extracellular matrix in diffuse
635 large B cell lymphoma. *Sci Rep.* 2016;6:30347.

636 12. Duan Z, Luo Y. Targeting macrophages in cancer immunotherapy. *Signal Transduct
637 Target Ther.* 2021;6(1):127.

638 13. Cassetta L, Pollard JW. Targeting macrophages: therapeutic approaches in cancer. *Nat
639 Rev Drug Discov.* 2018;17(12):887-904.

640 14. Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated
641 macrophages as treatment targets in oncology. *Nat Rev Clin Oncol.* 2017;14(7):399-416.

642 15. Panni RZ, Herndon JM, Zuo C, Hegde S, Hogg GD, Knolhoff BL, et al. Agonism of
643 CD11b reprograms innate immunity to sensitize pancreatic cancer to immunotherapies. *Science
644 translational medicine*. 2019;11(499).

645 16. Priceman SJ, Sung JL, Shaposhnik Z, Burton JB, Torres-Collado AX, Moughon DL, et al.
646 Targeting distinct tumor-infiltrating myeloid cells by inhibiting CSF-1 receptor: combating tumor
647 evasion of antiangiogenic therapy. *Blood*. 2010;115(7):1461-71.

648 17. Escamilla J, Schokrpur S, Liu C, Priceman SJ, Moughon D, Jiang Z, et al. CSF1
649 receptor targeting in prostate cancer reverses macrophage-mediated resistance to androgen
650 blockade therapy. *Cancer Res.* 2015;75(6):950-62.

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651 18. Jaynes JM, Sable R, Ronzetti M, Bautista W, Knotts Z, Abisoye-Ogunniyan A, et al.
652 Mannose receptor (CD206) activation in tumor-associated macrophages enhances adaptive
653 and innate antitumor immune responses. *Science translational medicine*. 2020;12(530).

654 19. Dangaj D, Abbott KL, Mookerjee A, Zhao A, Kirby PS, Sandaltzopoulos R, et al.
655 Mannose receptor (MR) engagement by mesothelin GPI anchor polarizes tumor-associated
656 macrophages and is blocked by anti-MR human recombinant antibody. *PLoS one*.
657 2011;6(12):e28386.

658 20. Tu MM, Abdel-Hafiz HA, Jones RT, Jean A, Hoff KJ, Duey JE, et al. Inhibition of the
659 CCL2 receptor, CCR2, enhances tumor response to immune checkpoint therapy. *Commun Biol*.
660 2020;3(1):720.

661 21. Strachan DC, Ruffell B, Oei Y, Bissell MJ, Coussens LM, Pryer N, et al. CSF1R
662 inhibition delays cervical and mammary tumor growth in murine models by attenuating the
663 turnover of tumor-associated macrophages and enhancing infiltration by CD8(+) T cells.
664 *Oncoimmunology*. 2013;2(12):e26968.

665 22. Zhu Y, Knolhoff BL, Meyer MA, Nywening TM, West BL, Luo J, et al. CSF1/CSF1R
666 blockade reprograms tumor-infiltrating macrophages and improves response to T-cell
667 checkpoint immunotherapy in pancreatic cancer models. *Cancer Res*. 2014;74(18):5057-69.

668 23. Neubert NJ, Schmittnaegel M, Bordry N, Nassiri S, Wald N, Martignier C, et al. T cell-
669 induced CSF1 promotes melanoma resistance to PD1 blockade. *Science translational medicine*.
670 2018;10(436).

671 24. Magkouta SF, Vaitis PC, Pappas AG, Iliopoulos M, Kostis CN, Psarros K, et al.
672 CSF1/CSF1R Axis Blockade Limits Mesothelioma and Enhances Efficiency of Anti-PDL1
673 Immunotherapy. *Cancers (Basel)*. 2021;13(11).

674 25. Cannarile MA, Weisser M, Jacob W, Jegg AM, Ries CH, Rüttinger D. Colony-stimulating
675 factor 1 receptor (CSF1R) inhibitors in cancer therapy. *Journal for immunotherapy of cancer*.
676 2017;5(1):53.

677 26. Diskin B, Adam S, Cassini MF, Sanchez G, Liria M, Aykut B, et al. PD-L1 engagement
678 on T cells promotes self-tolerance and suppression of neighboring macrophages and effector T
679 cells in cancer. *Nature immunology*. 2020;21(4):442-54.

680 27. Dong W, Wu X, Ma S, Wang Y, Nalin AP, Zhu Z, et al. The Mechanism of Anti-PD-L1
681 Antibody Efficacy against PD-L1-Negative Tumors Identifies NK Cells Expressing PD-L1 as a
682 Cytolytic Effector. *Cancer discovery*. 2019;9(10):1422-37.

683 28. Tang H, Liang Y, Anders RA, Taube JM, Qiu X, Mulgaonkar A, et al. PD-L1 on host cells
684 is essential for PD-L1 blockade-mediated tumor regression. *The Journal of clinical investigation*.
685 2018;128(2):580-8.

686 29. Tang F, Zheng P. Tumor cells versus host immune cells: whose PD-L1 contributes to
687 PD-1/PD-L1 blockade mediated cancer immunotherapy? *Cell Biosci*. 2018;8:34.

688 30. Hartley GP, Chow L, Ammons DT, Wheat WH, Dow SW. Programmed Cell Death
689 Ligand 1 (PD-L1) Signaling Regulates Macrophage Proliferation and Activation. *Cancer*
690 *Immunol Res*. 2018;6(10):1260-73.

691 31. Srivastava S, Furlan SN, Jaeger-Ruckstuhl CA, Sarvothama M, Berger C, Smythe KS, et
692 al. Immunogenic Chemotherapy Enhances Recruitment of CAR-T Cells to Lung Tumors and
693 Improves Antitumor Efficacy when Combined with Checkpoint Blockade. *Cancer cell*.
694 2021;39(2):193-208 e10.

695 32. Alizadeh D, Wong RA, Gholamin S, Maker M, Aftabizadeh M, Yang X, et al. IFN γ Is
696 Critical for CAR T Cell-Mediated Myeloid Activation and Induction of Endogenous Immunity.
697 *Cancer discovery*. 2021;11(9):2248-65.

698 33. Murad JP, Tilakawardane D, Park AK, Lopez LS, Young CA, Gibson J, et al. Pre-
699 conditioning modifies the TME to enhance solid tumor CAR T cell efficacy and endogenous
700 protective immunity. *Mol Ther*. 2021;29(7):2335-49.

701 34. Zarif JC, Hernandez JR, Verdone JE, Campbell SP, Drake CG, Pienta KJ. A phased
702 strategy to differentiate human CD14+monocytes into classically and alternatively activated
703 macrophages and dendritic cells. *Biotechniques*. 2016;61(1):33-41.

704 35. Priceman SJ, Gerdts EA, Tilakawardane D, Kennewick KT, Murad JP, Park AK, et al.
705 Co-stimulatory signaling determines tumor antigen sensitivity and persistence of CAR T cells
706 targeting PSCA+ metastatic prostate cancer. *Oncoimmunology*. 2018;7(2):e1380764.

707 36. Urak R, Walter M, Lim L, Wong CW, Budde LE, Thomas S, et al. Ex vivo Akt inhibition
708 promotes the generation of potent CD19CAR T cells for adoptive immunotherapy. *Journal for
709 immunotherapy of cancer*. 2017;5:26.

710 37. Wei Y, Zhao Q, Gao Z, Lao XM, Lin WM, Chen DP, et al. The local immune landscape
711 determines tumor PD-L1 heterogeneity and sensitivity to therapy. *The Journal of clinical
712 investigation*. 2019;130:3347-60.

713 38. Rongvaux A, Willinger T, Martinek J, Strowig T, Gearty SV, Teichmann LL, et al.
714 Development and function of human innate immune cells in a humanized mouse model. *Nature
715 biotechnology*. 2014;32(4):364-72.

716 39. Gordon SR, Maute RL, Dulken BW, Hutter G, George BM, McCracken MN, et al. PD-1
717 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity.
718 *Nature*. 2017;545(7655):495-9.

719 40. Zhang C, Rong HM, Li T, Zhai K, Tong ZH. PD-1 Deficiency Promotes Macrophage
720 Activation and T-Helper Cell Type 1/T-Helper Cell Type 17 Response in *Pneumocystis
721 pneumonia*. *Am J Respir Cell Mol Biol*. 2020;62(6):767-82.

722 41. Schaft N. The Landscape of CAR-T Cell Clinical Trials against Solid Tumors-A
723 Comprehensive Overview. *Cancers (Basel)*. 2020;12(9).

724 42. Bagley SJ, O'Rourke DM. Clinical investigation of CAR T cells for solid tumors: Lessons
725 learned and future directions. *Pharmacol Ther*. 2020;205:107419.

726 43. Pegram HJ, Lee JC, Hayman EG, Imperato GH, Tedder TF, Sadelain M, et al. Tumor-
727 targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior
728 conditioning. *Blood*. 2012;119(18):4133-41.

729 44. Mardiana S, Solomon BJ, Darcy PK, Beavis PA. Supercharging adoptive T cell therapy
730 to overcome solid tumor-induced immunosuppression. *Science translational medicine*.
731 2019;11(495).

732 45. Vignery A. Macrophage fusion: molecular mechanisms. *Methods in molecular biology*
733 (Clifton, NJ). 2008;475:149-61.

734 46. Genin M, Clement F, Fattacioli A, Raes M, Michiels C. M1 and M2 macrophages
735 derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. *BMC
736 Cancer*. 2015;15:577.

737 47. Kao JK, Wang SC, Ho LW, Huang SW, Lee CH, Lee MS, et al. M2-like polarization of
738 THP-1 monocyte-derived macrophages under chronic iron overload. *Ann Hematol*.
739 2020;99(3):431-41.

740 48. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate
741 alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome
742 biology*. 2013;14(4):R36.

743 49. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput
744 sequencing data. *Bioinformatics*. 2015;31(2):166-9.

745 50. Hsu F, Kent WJ, Clawson H, Kuhn RM, Diekhans M, Haussler D. The UCSC Known
746 Genes. *Bioinformatics*. 2006;22(9):1036-46.

747 51. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying
748 mammalian transcriptomes by RNA-Seq. *Nature methods*. 2008;5(7):621-8.

749 52. Lawrence M, Huber W, Pagès H, Aboyan P, Carlson M, Gentleman R, et al. Software
750 for computing and annotating genomic ranges. *PLoS Comput Biol*. 2013;9(8):e1003118.

PD-L1 blockade restores CAR T cell activity through IFN γ -regulation of CD163+ macrophages

751 53. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene
752 set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression
753 profiles. *Proceedings of the National Academy of Sciences of the United States of America*.
754 2005;102(43):15545-50.

755 54. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. The
756 Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst*.
757 2015;1(6):417-25.

758 55. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology:
759 tool for the unification of biology. *The Gene Ontology Consortium*. *Nat Genet*. 2000;25(1):25-9.
760

761 **Figure Legends**

762 **Figure 1: M2 macrophages suppress CAR T cells.** (a) Illustration of the immune-suppression
763 assay. CD14⁺ PBMCs were differentiated and polarized to M1 or M2 macrophages *in vitro*, and
764 macrophages, CAR T cells, and tumor cells were co-cultured and evaluated for functional
765 activities by flow cytometry. (b) Flow cytometry plots indicating the number of viable tumor cells
766 in each condition. (c, d) CAR T cell-mediated tumor cell killing of DU145-PSCA prostate cancer
767 (c) and CD19⁺ Daudi lymphoma (d) cells in the presence or absence of M1 or M2 macrophages
768 after 6 and 3 days, respectively. CAR T cell-mediated tumor cell killing was normalized to
769 untransduced (UTD) T cells. (e-h) Proliferation (10 days) (e), 4-1BB activation (6 days) (f, g),
770 and IFN γ secretion (3 days) (h) of T cells in the presence or absence of M1 or M2 macrophages
771 in the prostate cancer model. Proliferation and activation of T cells was measured by flow
772 cytometry. Secreted IFN γ in supernatant was measured by ELISA.

773

774 **Figure 2: CAR T cells alter M2 macrophage phenotypes.** (a) Illustration of the immune-
775 suppression assay to evaluate M2 macrophage phenotype. (b, c) Cell surface expression of
776 CD80 (b) and CD163 (c) in M2 macrophages in the immune-suppression assay evaluated by
777 flow cytometry. (d) Illustration of M2 macrophage stimulation with conditioned media (CM)
778 derived from CAR T cell:tumor cell co-cultures. (e, f) Cell surface expression of CD80 (e) and
779 CD163 (f) in M2 macrophages evaluated by flow cytometry 48 hours after CM stimulation. (g)
780 Transcriptional changes by bulk RNA-seq induced in M2 macrophages upon stimulation with
781 CAR T cell-derived CM. Expression of selected immune-related genes is shown relative to a
782 control condition stimulated with UTD T cell-derived CM. (h) Gene ontology (GO) enrichment
783 analysis highlighting activated immune-related biological pathways in M2 macrophages upon
784 stimulation with CAR T cell-derived CM.

785

PD-L1 blockade restores CAR T cell activity through IFN γ -regulation of CD163 $^{+}$ macrophages

786 **Figure 3: CAR T cells induce PD-L1 expression in M2 macrophages.** (a-c) PD-L1
787 expression in macrophages and DU145-PSCA tumor cells in the immune-suppression assay.
788 (d, e) Immunostaining of CD68 and PD-L1 in a humanized MISTRG mouse model following
789 CAR T cell therapy against subcutaneous DU145-PSCA (d) and intratibial LAPC9 (e) prostate
790 xenografts. (f, g) PD-L1 induction at the protein (f) and mRNA (g) levels following inhibition of
791 IFN γ signaling. Anti-IFN γ R1 antibody was used to block IFN γ signaling in the presence of
792 recombinant IFN γ or CAR T cell-derived CM collected from the tumor cell killing assay. (h) PD-
793 L1 induction following inhibition of various signaling pathways. CAR T cell-derived CM was
794 applied to M2 macrophages in the presence of various small molecule inhibitors: fludarabine
795 (STAT1 i), C188-9 (STAT3 i), itacitinib (JAK1 i), AG490 (JAK2 i), AZD1480 (JAK1/2 i), Bay11-
796 7082 (NF κ B i), Akti VIII (AKT i), CZC24832 (PI3K i), rapamycin (mTOR i). PD-L1 induction was
797 evaluated by flow cytometry 48 hours after CM stimulation.

798

799 **Figure 4: PD-L1 blockade restores CAR T cell function in the presence of suppressive M2**
800 **macrophages.** CAR T cell function was evaluated in the immune-suppression assay in the
801 presence of PD-L1 blockade. (a) Flow cytometry plots indicating the number of viable tumor
802 cells in each condition in the presence or absence of anti-PD-L1 antibody, Atezolizumab
803 (Atezo). (b) Quantification of CAR T cell-mediated killing of DU145-PSCA tumor cells in the
804 presence or absence of Atezo. (c) 4-1BB T cell activation was evaluated by flow cytometry. (d)
805 IFN γ secretion was measured by ELISA. (e) Tumor cell killing of CAR T cells in the presence or
806 absence of two clinically approved anti-PD-L1 antibodies, Atezo and Avelumab (Ave).

807

808 **Figure 5: Combination of PD-L1 blockade and CAR T cell therapy depletes M2**
809 **macrophages via IFN γ signaling.** (a, b) Analysis of M2 macrophages in the immune-
810 suppression assay in the presence or absence of PD-L1 blockade. (c, d) Analysis of M2

811 macrophages stimulated with CAR T cell-derived CM in the presence or absence of PD-1 or
812 PD-L1 blockade. (e-g) Images and analysis of M2 macrophage stimulated with CAR T cell-
813 derived CM in the presence or absence of PD-L1 and/or IFN γ R1 blockade. (f, g) The number of
814 total viable M2 macrophages (a, c, f) and the frequency and number of CD163 $^{+}$ M2
815 macrophages (b, d, g) were evaluated by flow cytometry.

Figure 1

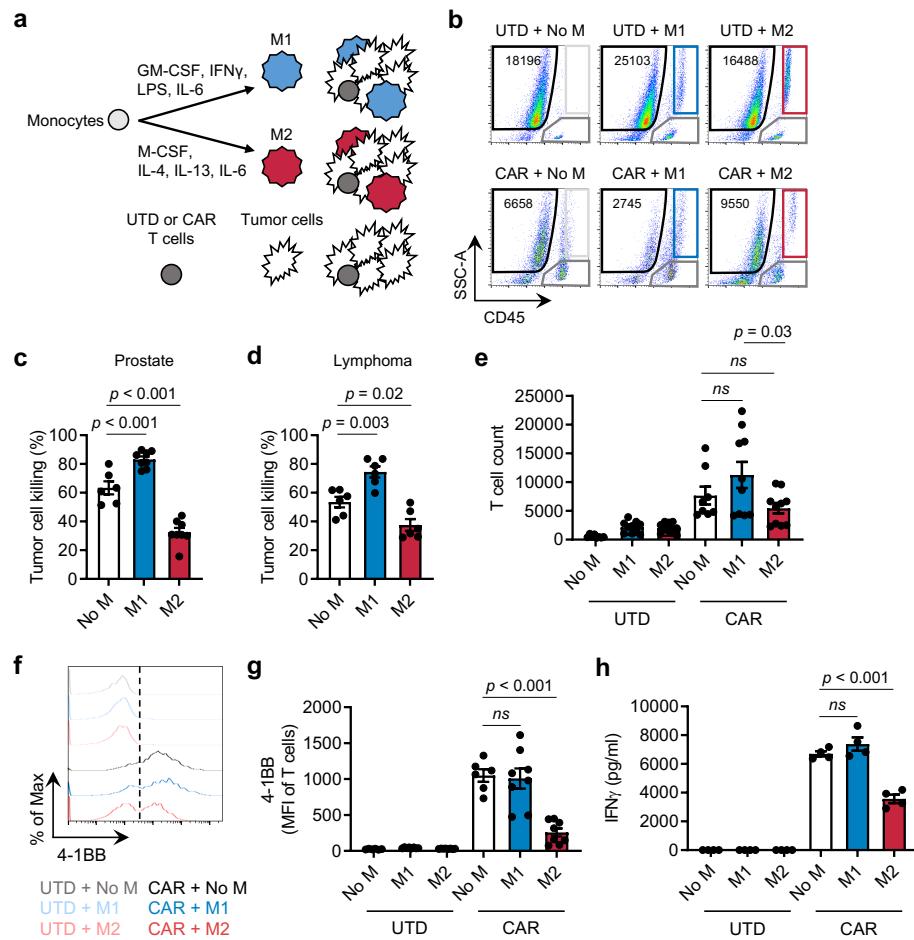


Figure 2

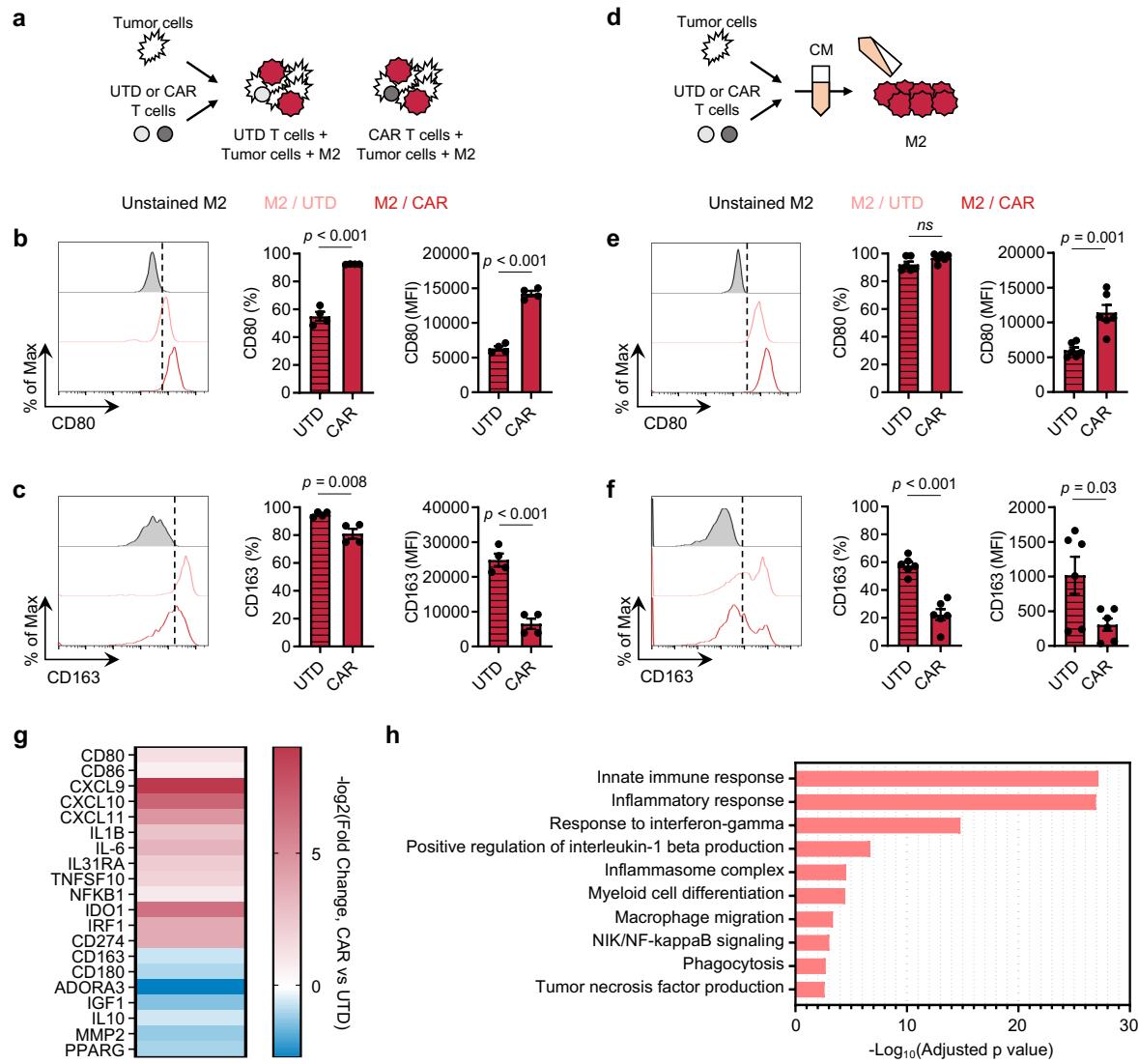


Figure 3

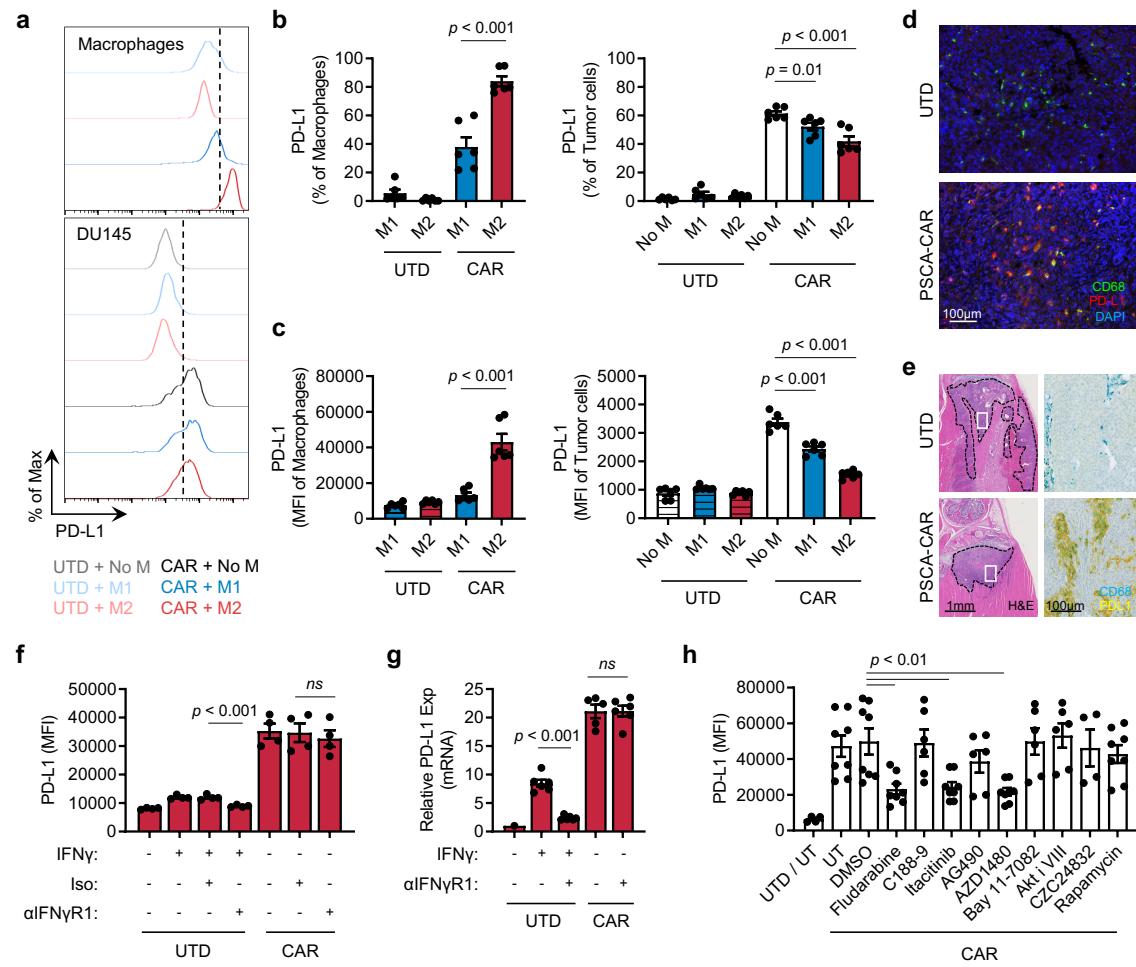


Figure 4

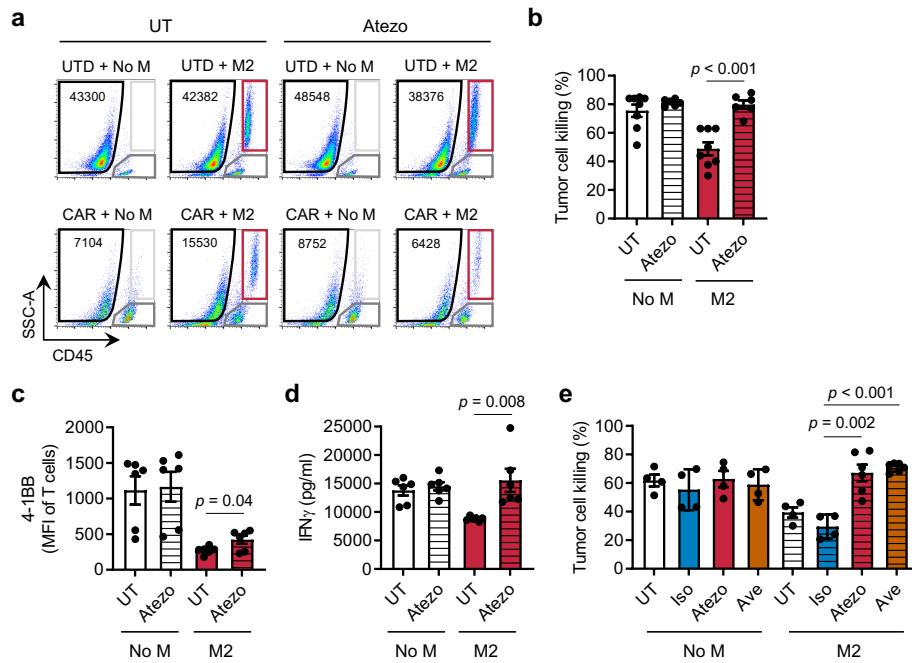


Figure 5

