Neoadjuvant androgen deprivation therapy with or without Fc-enhanced non-fucosylated anti-CTLA-4 (BMS-986218) in high risk localized prostate cancer: a randomized phase 1 trial

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Abstract

1   Men with high-risk localized prostate cancer exhibit high rates of post-surgical

2   recurrence. In these patients, androgen deprivation therapy (ADT) is

3   immunomodulatory, however increased infiltration of regulatory T cells (Tregs) may limit

4   the antitumor immune effects of ADT. We designed a neoadjuvant clinical trial to test

5   whether BMS-986218 – a next-generation non-fucosylated anti-CTLA-4 antibody

6   engineered for enhanced antibody-dependent cellular cytotoxicity or phagocytosis

7   (ADCC/P) – depletes intratumoral Tregs and augments the response to ADT. In this

8   single-center, two-arm, open-label study, 24 men with high-risk localized prostate

9   cancer were randomized to receive a single dose of ADT with or without two pre-

10   operative doses of BMS-986218 (anti-CTLA4-NF) prior to radical prostatectomy.

11   Treatment was well tolerated and feasible in the neoadjuvant setting. A secondary

12   clinical outcome was the rate of disease recurrence, which was lower than predicted in

13   both arms. Mechanistically, anti-CTLA4-NF reduced ADT-induced Treg accumulation

14   through engagement of CD16a/*FCGR3A* on tumor macrophages, and depth of Treg

15   depletion was quantitatively associated with clinical outcome. Increased intratumoral

16   dendritic cell (DC) frequencies also associated with lack of recurrence, and pre-clinical

17   data suggest ADCC/P-competent anti-CTLA-4 antibodies elicit activation and expansion

18   of tumor DCs. Patients receiving anti-CTLA4-NF also exhibited phenotypic signatures of

19   enhanced antitumor T cell priming. In total, this study provides the first-in-human

20   evidence of Treg depletion by glycoengineered antibodies targeting CTLA-4 in humans

21   and their potential in combination with ADT in prostate cancer patients with high-risk of

22   recurrence.

Introduction

To date, immune checkpoint blockade immunotherapy (ICB) has proven largely ineffective for patients with prostate cancer (PCa)[1]. Agents targeting the PD-1/PD-L1 pathway alone or in combination with androgen receptor inhibitors (KEYNOTE-164[2], KEYNOTE-991[3], IMbassador250[4]), chemotherapy (KEYNOTE-921[5]), or PARP inhibition (KEYLYNK-010[6]) have failed in phase III trials. Similarly, CTLA-4 blockade in a chemo-naïve population (Ipi-095[7]) or post-docetaxel in combination with bone-targeted radiation (Ipi-043[8]) did not meet pre-specified endpoints. However, long-term follow-up on the Ipi-043 trial indicates a potential survival advantage of ipilimumab versus placebo[8]. Furthermore, a phase II study of combination ipilimumab and nivolumab in metastatic patients pre- or post-chemotherapy (CheckMate-650[9]) showed a survival signal as compared to historical anti-PD-(L)1 monotherapy controls[9]. These findings suggest that CTLA-4 blockade may possess clinical activity in PCa that warrants further investigation, particularly if concerns regarding dose-dependent toxicity can be addressed

One hypothesis explaining the relatively low efficacy of ICB in PCa is that baseline T cell infiltration is insufficient for ICB to elicit robust T cell-mediated antitumor immunity[1]. Interestingly, our prior findings showed that standard of care androgen deprivation therapy (ADT) initially increases CD8 T cell infiltration into primary PCa in both murine models and patients[10,11]. However, a counter-regulatory influx of immunosuppressive $CD4^+FoxP3^+$ regulatory T cells (Tregs) inhibits the inflammatory effects of ADT, implicating adaptive Treg resistance as a critical inhibitory mechanism[10,11]. As such, depletion of tumor-infiltrating Tregs (TI-Tregs) by anti-CTLA-4 antibodies with enhanced

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47   antibody dependent cellular cytotoxicity or phagocytosis (ADCC/P) extends depth and

48   duration of response to androgen deprivation (ADT) in murine models, whereas non-

49   depleting anti-CTLA-4 antibodies and PD-1 blockade in the same setting is ineffective[10].

50   These Fc-enhanced agents have also been hypothesized to have an improved

51   therapeutic index[12]. Therefore, we reasoned that inhibiting TI-Treg number and/or

52   function in combination with ADT may effectively potentiate ICB efficacy in PCa.

53   Methods for depleting TI-Tregs have been studied thoroughly in pre-clinical models but

54   have as yet not been successfully validated in the clinic. Antibodies targeting cell

55   surface proteins enriched on TI-Tregs such as CTLA-4, CD25, CCR4, TIGIT, and CCR8

56   have either failed to show Treg depletion activity and/or efficacy in clinical trials or

57   remain under investigation[13-16]. We thus sought to further investigate anti-CTLA-4 in this

58   context, given its potential clinical activity in PCa[8] and the fact that the first-generation

59   anti-CTLA-4 antibodies ipilimumab and tremelimumab were not optimized for Treg

60   depletion. Tremelimumab is a human IgG2 antibody with relatively low affinity for the

61   FcγRs such as CD16a (*FCGR3A*) that mediate ADCC/P, while Ipilimumab is a human

62   IgG1 isotype that can elicit ADCC/P. However, existing clinical data suggest ipilimumab

63   is a weak depleter, with evidence of Treg depletion restricted to patients with a high-

64   affinity germline polymorphic variant of *FCGR3A* (V158F)[16,17]. To overcome this

65   limitation, antibodies targeting CTLA-4 bearing non-fucosylated Fc regions have been

66   recently developed to enhance binding to CD16a/*FCGRIIIA*, resulting in enhanced

67   ADCP/ADCC[18]. However, it remains unclear whether non-fucosylated anti-CTLA-4

68   antibodies may be more effective in depleting TI-Tregs in patients. Furthermore,

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69   whether these next-generation Fc-enhanced antibodies may overcome adaptive Treg

70   resistance following ADT is also unknown.

71   To address this challenge, we designed a randomized neoadjuvant clinical trial to

72   evaluate the safety, feasibility, clinical efficacy, and immunological activity of non-

73   fucosylated ipilimumab (BMS-986218; hereafter anti-CTLA4-NF) with ADT versus ADT

74   alone prior to surgery in men with high-risk localized prostate cancer (NeoRED-P;

75   NCT04301414). This window-of-opportunity trial enabled careful interrogation of the

76   immunological mechanisms of response to anti-CTLA4-NF through orthogonal

77   immunoprofiling by single cell RNA sequencing (scRNAseq), mass cytometry (CyTOF),

78   and spatial proteomics (multiplex immunofluorescence). We also leveraged the novel

79   RNA-seq-based pipeline PISCES to perform robust protein activity level assessment of

80   tumor microenvironment subpopulations and of their drug-mediated depletion, thus

81   bridging the transcriptomic and proteomic datasets. Pre-clinical validation studies in the

82   syngeneic MycCaP PCa model closely mirrored observed immunological correlates of

83   clinical response. Our findings demonstrate the tolerability and promising clinical activity

84   of ADT plus anti-CTLA4-NF in the neoadjuvant setting and provide a first-in-human

85   examination of whether Fc-engineered non-fucosylated anti-CTLA4 antibody variants

86   mediate TI-Treg depletion, while identifying additional putative mechanisms by which

87   anti-CTLA4-NF antibodies may augment antitumor immune responses in PCa.

Results

Treatment Safety, Feasibility and Efficacy as Neoadjuvant Therapy in Prostate Cancer

Between February 26, 2020, and November 15, 2022, 24 patients were enrolled at Columbia University Irving Medical Center. The first four patients received ADT with degarelix acetate + anti-CTLA4-NF as a safety lead-in, after which 20 patients were randomized 1:1 to ADT-only or ADT + anti-CTLA4-NF arms (Figure 1A). One patient in the ADT + anti-CTLA4-NF arm did not receive surgery due to operating room closure during SARS-CoV2 pandemic, and one patient in each arm was lost to follow-up (Figure 1B). For correlative studies, samples from a cohort of twelve stage- and grade-matched treatment-naïve patients were collected as untreated controls. Baseline characteristics of trial patients are summarized in Table 1. The arms were well balanced with respect to age, race and ethnicity, pre-treatment serum PSA or testosterone, and rate of nodal involvement or surgical margin involvement. Patients in the ADT + anti-CTLA4-NF arm trended towards modestly higher grade group 5 disease and higher predicted recurrence risk as measured by a validated risk nomogram (see Methods) than the ADT alone arm. Tumor exome and mutational profiling of all patient-derived samples showed 12 patients with AR-V7 splice variants (38%), eight patients exhibiting TMRPSS2:ERG fusion events (25%), four patients bearing mutated *TP53* (13%)*,* three patients with copy number loss or mutations in *PTEN* (9%), and two patients each with alterations *in APC* and *CTNNB1* (6%; Extended Data Figure 1A). Of note, a single non-recurring patient in the ADT + anti-CTLA4-NF arm was found to exhibit high tumor mutational burden (TMB-H; 26 mutations/Mb), associated with a germline mutation in *MSH2*.

112   Treatment related adverse advents (TRAE) of any grade were observed in 80% of ADT-

113   only patients and 71% of ADT + anti-CTLA4-NF patients, the most common events

114   were injection site reactions in both arms (Table 2). Three Grade 1-2 gastrointestinal

115   events were observed in patients receiving anti-CTLA4-NF. Severe treatment-related

116   adverse events (Grade ≥ 3) were rare; a single patient in the ADT + anti-CTLA4-NF arm

117   exhibited a Grade 3 asymptomatic serum lipase elevation that resolved without

118   additional therapy and did not result in treatment discontinuation. No unexpected

119   surgical complications were observed, supporting a tolerable safety profile for the

120   addition of anti-CTLA4-NF to ADT in this patient population.

121   In the ADT-only arm, 80% of patients had an undetectable PSA at 12 months, with a

122   median recurrence-free survival of 1.61 years (IQR: 1.50–1.99 years) (Table 3). In the

123   ADT + anti-CTLA4-NF arm, 75% had an undetectable PSA at 12 months, with a median

124   recurrence-free survival of 1.82 years (IQR: 0.89–2.07 years) (Table 3). The overall

125   recurrence rate in patients treated with ADT + anti-CTLA4-NF was 29% (4 of 12

126   evaluable patients) and 20% (2 of 10 patients) in the ADT alone arm (Table 3). This

127   study was not powered to compare efficacy between treatment arms. However, after

128   predicting 2-year recurrence rates on a per-patient basis using a validated clinical risk

129   nomogram (Memorial Sloan Kettering Cancer Center Post-Radical Prostatectomy

130   Prediction; see Methods), we found both arms exceeded predicted 2-year recurrence-

131   free survival rates (Figure 1C-D). Patients in the ADT-only arm had a PSA50 response

132   rate prior to surgery of 33%, while patients in the ADT + anti-CTLA4-NF arm had a

133   PSA50 response rate of 54% (p=0.4; Table 3; Figure 1E-F). All patients recovered

134   testosterone to baseline levels within 6 months of prostatectomy (Extended Data Figure

135   2). Overall, these data suggest adding anti-CTLA4-NF to ADT is well-tolerated and

136   feasible in the neoadjuvant setting for patients with high-risk localized PCa.

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138   **Anti-CTLA4-NF suppresses ADT-induced Treg expansion**

139   To elucidate the effects of ADT + anti-CTLA4-NF versus ADT alone versus no treatment

140   on prostate tumor-infiltrating immune populations, we performed transcriptomic and

141   proteomic-based immune profiling via paired scRNA sequencing (via 10X Genomics

142   droplet-based platform), mass cytometry (CyTOF), and immunofluorescence (IF; Figure

143   2A). Of the 36 patients assessed, 26 had scRNAseq data of sufficient quality, 25 had

144   CyTOF data of sufficient quality, and 29 had IF data of sufficient quality to be included in

145   further analyses (see Extended Data Figure 3A and methods for QC criteria and Figure

146   2B for a representative schematic). We first tested our secondary endpoint hypothesis

147   that anti-CTLA4-NF reduces TI-Treg density in ADT-treated PCa tumors by quantifying

148   the number of CD4$^+$FoxP3$^+$ Tregs per mm^2 in whole-tissue FFPE slides by IF (see

149   representative staining images in Figure 2C). For this analysis, we censored one patient

150   with MSI^{hi} status given the known association between MSI^{hi} status, high TMB, and

151   increased overall tumor T cell infiltration relative to MSI^{WT} patients[19]. In line with our

152   previous findings, we found ADT significantly increases Treg densities in the PCa tumor

153   parenchyma (p=0.001; Figure 2D). Concurrent treatment with ADT and anti-CTLA4-NF

154   resulted in a strong trend towards reduction in Treg density (p=0.06), indicating anti-

155   CTLA4-NF may have measurable Treg depletion activity.

156   To validate these results, we interrogated the immune TME of this cohort by scRNAseq

157   and CyTOF performed on independent, freshly processed prostatectomy specimens.

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158   For scRNAseq data analysis we utilized the novel PISCES pipeline incorporating

159   unbiased Louvain-based clustering and metaVIPER (virtual inference of protein activity

160   by enriched regulon analysis[20]) to quantitatively infer activity of approximately 6,500

161   regulatory proteins per cell[21]. Based on CITE-seq analysis and experimental validation,

162   this approach has been shown to outperform traditional gene expression-based

163   methods, wherein RNA dropout limits detection of key lineage- and function-determining

164   transcripts and reduces cluster resolution (see Methods for more detail and prior

165   studies[21-23] for pipeline validation). Integrating cells from all patients, we stratified

166   immune metaclusters corresponding to immune lineages including T/NK cells, B cells,

167   macrophage/DCs, monocytes, neutrophils, and plasma cells by PISCES and cross-

168   validated cluster identity at the gene expression level by parallel SingleR[24] and

169   supervised annotation (Figure 2E-F). Additionally, we identified stromal cell clusters

170   including fibroblasts, endothelial cells, megakaryocyte-erythroid progenitors (MEPs),

171   club/glandular cells, and four molecularly distinct epithelial tumor clusters. Tumor cell

172   clusters were validated by aberrant copy number analysis, using inferCNV[25,26]

173   (Extended Data Figure 3B). In parallel, we performed semi-supervised FlowSOM[27]

174   clustering followed by supervised UMAP embedding on all live CD45+ events from the

175   CyTOF dataset (Extended Data Figure 4A; see methods for workflow details). We

176   clearly resolved multiple immune populations using established lineage-defining protein

177   markers, including multiple T cell subsets, NK cells, monocytes, macrophage/DCs, and

178   neutrophils (Figure 2G-H). Further validating the PISCES-based stratification,

179   comparative analysis of immune lineages defined by both platforms revealed significant

180   immune subpopulation fraction correlations between scRNAseq and CyTOF for all

181   immune lineages on a per-patient basis. NK cells were a notable exception and were

182   not significantly correlated across platforms (Extended Data Fig 4B). Though correlated,

183   neutrophils were comparably underrepresented in the scRNAseq data relative to

184   CyTOF, as is common in droplet-based scRNAseq methodologies (Extended Data

185   Figure 3C-D). These data overall confirm faithful capture and annotation of relevant cell

186   types by both scRNAseq and CyTOF.

187   To test the hypothesis that anti-CTLA4-NF mediates intratumoral Treg depletion *in vivo*,

188   we first subclustered the scRNAseq T/NK cell metacluster to clearly resolve a Treg

189   cluster as validated by enrichment of established Treg transcriptional signature scores,

190   inferred activity of Treg lineage marker proteins by VIPER, and SingleR annotation

191   (Figure 2I-K). Mirroring the IF data, patients receiving ADT + anti-CTLA4-NF exhibited a

192   strong trending decrease in Treg frequency as compared to ADT alone (p=0.06; Figure

193   2L). In the CyTOF dataset, Tregs were clearly identified at the protein level in a

194   FlowSOM-derived cluster expressing the canonical $CD4^+CD25^+CD127^-FoxP3^+$

195   phenotype (Figure 2M) and additional markers indicative of tumor-infiltrating Tregs

196   including Helios, CCR8, TIGIT, CD39, and CCR4 (Figure 2N). Consistent with the

197   PISCES analyses and IF results, we observed a non-significant but consistent trend

198   towards a reduction in Treg frequency in the ADT + anti-CTLA4-NF arm compared to

199   the ADT alone arm (Figure 2O; p=0.07). Though the traditional significance threshold

200   was not reached by individual modalities, these data are highly consistent across three

201   independent immune profiling platforms in a small, prospectively treated patient cohort,

202   and thus suggest non-fucosylated anti-CTLA-4 antibody BMS-986218 may reduce

203   intratumoral Treg frequencies in prostate cancer.

Increased depletion of phenotypically activated Tregs associates with clinical response

206 As our data suggests Treg depletion via anti-CTLA4-NF is limited in depth, we sought to

207 further validate and investigate the biological activity of anti-CTLA4-NF by interrogating

208 the molecular phenotype of residual Tregs in post-treatment tumors. We utilized the

209 CyTOF data for this analysis, as we captured an insufficient number of Tregs by

210 scRNAseq to permit rigorous subclustering of the TI-Treg compartment. Semi-

211 supervised FlowSOM subclustering and PaCMAP embedding of TI-Tregs in the CyTOF

212 dataset identified nine phenotypically distinct Treg subpopulations. These were primarily

213 resolved by differential expression of CD45RO, Ki67, granzyme B, CD39, CCR8, HLA-

214 DR, and/or 4-1BB (Figure 3A-C). This analysis revealed a potential phenotypic shift in

215 residual Tregs for patients treated with anti-CTLA4-NF, including a decrease in Tregs in

216 cluster Tr.5 defined by low CD39 expression, and increase in Tregs in cluster Tr.9, as

217 defined by high expression of 4-1BB and other activation markers (Figure 3D-E). To

218 validate these observations, we measured the fold change in geometric MFI (gMFI) of

219 all phenotypic markers in our CyTOF panel between Tregs in patients treated with ADT

220 versus patients treated with anti-CTLA4-NF (Figure 3F). We found Tregs remaining in

221 tumors after anti-CTLA4-NF exposure expressed the activation markers 4-1BB, CD39,

222 CCR8, CTLA-4, and CD25, suggesting that tumor-infiltrating Tregs remaining after anti-

223 CTLA4-NF are enriched for an activated phenotype (Figure 3F).

224 Given this observation, we next examined whether the depth of TI-Treg depletion

225 observed in treated patients associated with clinical response. For this, we stratified

226 patients that received ADT + anti-CTLA4-NF by recurrence (n=4) versus non-recurrence

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227   (n=7) according to clinically annotated PSA recurrence within 2 years following surgery.

228   We limited this analysis to the scRNAseq dataset, as we did not capture CyTOF data for

229   all patients that recurred. We found that TI-Treg frequencies at time of surgery were

230   significantly higher in patients that went on to recur as compared to those that did not

231   recur (p=0.03; Figure 3G). In contrast, no other lymphocyte subset was significantly

232   associated with clinical outcome (Extended Data Figure 4E). In addition, we stratified

233   patients that received ADT + anti-CTLA4-NF by TI-Treg frequency and found that

234   patients with high TI-Treg frequencies exhibited significantly shorter time-to-recurrence

235   as compared to patients with low TI-Treg frequencies (p=0.0008; Figure 3H). There

236   were insufficient recurrence events in the ADT alone arm to perform a statistical

237   analysis of time-to-recurrence in ADT-treated patients stratified by TI-Treg frequency,

238   though no trend was observed (Extended Data Figure 4F). In agreement with this data,

239   it is unlikely TI-Treg frequency in absence of anti-CTLA4-NF would associate with

240   recurrence in this setting as prior studies show no association between Treg frequency

241   and recurrence risk in localized PCa[28]. These correlative findings thus suggest that

242   depth of TI-Treg depletion is associated with response to ADT + anti-CTLA4-NF in

243   patients with high-risk localized prostate cancer.

244   **Efficiency of myeloid-mediated Treg depletion associates with duration of**

245   **response to therapy**

246   Given the putative association between anti-CTLA4-NF-mediated TI-Treg depletion and

247   clinical response, we next sought to investigate potential mechanisms for TI-Treg

248   depletion. In pre-clinical models, antibody-mediated Treg depletion by anti-CTLA4

249   canonically requires interactions between the anti-CTLA4 Fc region and activating

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250   FcγRs such as CD16a (*FCGR3A*) on tumor-associated macrophages (TAMs)[29,30]. To

251   determine whether macrophage FcγR expression was associated with Treg depletion

252   efficiency in response to ADT + anti-CTLA4-NF, we first annotated macrophage

253   populations in the scRNAseq dataset by both supervised manual annotation and

254   through systematic testing of established prostate cancer macrophage gene expression

255   signatures[31] (Figure 4A-C). We identified 3 putative macrophage subsets: a recently

256   described prostate-specific MT$^+$ macrophage subset[31] and two subsets of TAMs defined

257   by TREM2 and CD206 (*MRC1)*, respectively. Of these, TREM2$^+$ and CD206$^+$ TAMs

258   exhibited highest predicted protein activity for multiple FcγRs (Figure 4D), and TREM2$^+$

259   TAMs showed the highest activity of the activating FcγR CD16a/*FCGR3A* (Figure 4E).

260   To test the hypothesis that anti-CTLA4-NF depletes Tregs through CD16a/*FCGR3A* on

261   TAMs, we examined whether CD16a/*FCGR3A* protein activity was correlated with

262   intratumoral TI-Treg frequencies on a per-patient basis. We found predicted

263   CD16a/*FCGR3A* protein activity across all myeloid subsets to be significantly correlated

264   with reduced Treg frequencies in ADT + anti-CTLA4-NF patients (p=0.033; Figure 4F).

265   In contrast, we found no significant correlation between myeloid CD16a/*FCGR3A*

266   protein activity and TI-Treg frequencies in patients who received ADT alone (p=0.223;

267   Figure 4F), supporting the hypothesis that CD16a/*FCGR3A* mediates TI-Treg depletion

268   in the context of treatment with anti-CTLA4-NF. We also found similar correlations

269   between TI-Treg frequencies and CD16a/*FCGR3A* activity within the TREM2$^+$ TAMs

270   specifically (Figure 4G). These findings support the notion that TI-Treg depletion by anti-

271   CTLA4-NF is at least partially mediated through canonical tumor macrophage FcγR

272   interactions in the human PCa tumor microenvironment.

Intratumoral dendritic cell frequencies associate with response to ADT + anti-CTLA4-NF

Overall, myeloid frequencies were not significantly altered by treatment (Figure 4H).

After stratification of ADT + anti-CTLA4-NF patients by recurrence status, however, we

observed intratumoral dendritic cell (DC) frequencies at time of surgery trended towards

higher abundance in patients that did not recur (p=0.06; Figure 4I). We thus stratified

patients by DC frequency at time of surgery and found those with fewer tumor-infiltrating

DCs exhibited a significantly shorter time to recurrence (p=0.029; Figure 4J). To further

investigate this novel association between DC frequencies and anti-CTLA4-NF

response, we returned to the MycCaP pre-clinical model of castration-sensitive prostate

cancer where we previously assessed mechanistic effects of ADCC-competent versus

ADCC-incompetent anti-CTLA4 antibodies in combination with ADT[10]. We implanted 8–

10-week-old male FVB mice with syngeneic Myc-CaP prostate tumors and performed

chemical castration with degarelix acetate (ADT) when tumors averaged ~200mm^3 in

volume (Figure 4K). Mice received a single dose of ADT alone or in combination with

three doses of anti-CTLA-4 (clone 9D9; 5 mg/kg) administered IP at three-day intervals.

To confirm Treg depletion was mediated through FcγR-dependent ADCC, mice

received either Fc-competent antibody (mIgG2a; anti-CTLA4 [D]) or the equivalent

clone and isotype bearing a mutation that nullifies FcγR interactions[32] (mIgG2a-

LALAPG; anti-CTLA4 [ND]). These data were consistent with our prior findings that anti-

CTLA4 (D), but not anti-CTLA4 (ND), augments response to ADT in the Myc-CaP model

(Figure 4L-M). Following treatment, tumors and draining lymph nodes were harvested

and profiled by 45-parameter spectral flow cytometry, and data was analyzed by semi-

supervised FlowSOM clustering and supervised UMAP or unsupervised PaCMAP

298   embedding for visualization (Figure 4N; Extended Data Fig 5A-B). Consistent with

299   patient data, ADT + anti-CTLA4 (D) significantly reduced Treg frequencies compared to

300   ADT and ADT + anti-CTLA4 (ND) groups, indicating robust antibody-dependent Treg

301   depletion (p=0.0031; Figure 4O, top). We further found that animals treated with ADT +

302   anti-CTLA4 (D) exhibited significantly greater frequencies of tumor-infiltrating CD11c$^+$

303   MHC-II^{hi} DCs compared to those receiving ADT or ADT + anti-CTLA4 (ND) (p=0.0009;

304   Figure 4O). Systematic subclustering of the tumor DC compartment revealed a striking

305   phenotypic shift in DCs in response to anti-CTLA4 (D) (Figure 4P, Extended Data Fig

306   5C-D). While DC phenotypes in mice treated with ADT or ADT + anti-CTLA4 (ND) were

307   enriched for canonical cDC1 and cDC2 markers such as CD103 or CD11b, respectively,

308   nearly all DCs in mice treated with anti-CTLA-4 (D) expressed PD-L2, a marker of the

309   DC3 subset and/or mRegDC (mature DC enriched in immunoregulatory molecules)

310   program. The mRegDC phenotype is induced upon tumor antigen uptake, and

311   mRegDCs are thought to possess a unique role in shaping antitumor immune

312   responses[33]. The increase in PD-L2$^+$ DCs was unique to mice receiving anti-CTLA4 (D)

313   (Figure 4Q, Extended Data Fig 5E). Taken together, these data indicate ADCC/P

314   competent antibodies targeting CTLA-4 modulate tumor-infiltrating DCs, and that an

315   increased DC frequency following treatment with anti-CTLA4-NF is associated with

316   positive clinical outcome.

317

318   **ADT + anti-CTLA-4-NF enhances antitumor T cell priming**

319   Given the observed effects of anti-CTLA4-NF therapy on tumor Treg frequencies and

320   myeloid phenotypes, we next sought to interrogate the effect of ADT + anti-CTLA4-NF

321   on CD8 T cell responses in the tumor. For enhanced resolution of CD8 phenotypes in

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322   the scRNAseq data, we first refined the previously compiled CD8-1 and CD8-2 clusters

323   (Figure 2K), removing a contaminating cell population presenting low *Cd8a* expression,

324   moderate *Cd4* expression, and an otherwise naïve gene signature (Extended Data Fig

325   6A-B). Subcluster analysis of the curated CD8 T cells resulted in an optimal three

326   cluster solution (Figure 5A). Utilizing established pan-cancer CD8 T cell gene signatures

327   from Zheng *et al.*[34] and prostate CD8 T cell signatures from Tuong *et al.*[31] we annotated

328   these clusters as central memory (IL7R$^+$ memory signature enrichment, prostate CD8

329   memory T cell signature enrichment, expression of CCR7), effector memory

330   (enrichment of GZMK$^+$ early TEM signature), and effector/resident memory CD8 T cells

331   (enrichment of prostate CD8 effector T cell signature, expression of CD103, GzmB,

332   TOX2; Figure 6B). These subsets were not significantly altered by ADT or ADT + anti-

333   CTLA4-NF. To further interrogate tumor-infiltrating CD8 T cell phenotypes by CyTOF,

334   we used semi-supervised FlowSOM clustering to define eight CD8 T cell subclusters

335   (Figure 6D). While these clusters did not map precisely onto the three cluster solution in

336   the scRNAseq data, we did observe clusters that most closely matched the effector

337   memory (Tc.4; CD45RA$^-$CD45RO$^+$CD27$^+$HLA-DR$^+$PD-1^{mid}) phenotype and cells that

338   matched the effector/resident memory (Tc.6; CD45RO$^-$HLA-DR$^+$CTLA-4$^+$TIGIT$^+$GzmB$^+$)

339   phenotype that mirrored non-significant trends in the scRNAseq data after anti-CTLA4-

340   NF (Extended Data Fig 6C-D). Notably, we also observed a cluster with clear

341   enrichment in a subset of patients receiving ADT + anti-CTLA4-NF defined by 4-1BB

342   and CD39 (Figure 5E-G), markers that are associated with recently primed and tumor-

343   specific T cells[35,36]. We found the dual 4-1BB$^+$CD39$^+$ phenotype to also define a distinct

344   subset of CD4$^+$FoxP3$^-$ conventional CD4 T cells (Tconv) that were uniquely present in a

16

345 subset of patients receiving ADT + anti-CTLA4-NF (Figure 5H-K, Extended Data 6E-F).

346 These data suggest anti-CTLA4-NF may enhance priming of tumor-specific T cell

347 populations, either in concert with or independent of Treg depletion. To test whether this

348 effect requires FcγR interactions, we returned to our murine model where we profiled

349 effects of anti-CTLA4 (D) versus anti-CTLA4 (ND) in combination with ADT. We

350 observed a distinct cluster of 4-1BB$^+$CD39$^+$ CD8 T cells in Myc-CaP tumors that were

351 uniquely induced by ADT + anti-CTLA4 (D) and were absent in ADT-only and ADT +

352 anti-CTLA4 (ND) groups (Figure 5L-O, Extended Data Fig 6G-I), indicating FcγR

353 engagement is required for induction of the 4-1BB$^+$CD39$^+$ CD8 T cell phenotype under

354 anti-CTLA4 therapy. Supporting our hypothesis that these cells represent a recently

355 primed population, we found a significant increase in CD8 and CD4 Tconv cells bearing

356 phenotypic markers of recent priming (CD44$^+$Ki67$^+$) in tumor draining LNs (tdLNs) in

357 mice receiving anti-CTLA4 (D) relative to anti-CTLA4 (ND) or ADT alone (Figure 5P-R,

358 Extended Data Fig 6J). This was not associated with Treg depletion in the tdLNs, but

359 rather an increase in Treg frequency in the tdLNs as has been previously shown in the

360 context of anti-CTLA4 therapeutics[37] (p<0.0001; Extended Data Figure 6K). These

361 findings support an additional putative mechanism of action of Fc engineered anti-

362 CTLA4-NF antibodies, potentially distinct from Treg depletion, involving enhanced

363 priming of tumor-specific CD8 and/or non-Treg CD4 Tconv cells.

364   **Discussion**

365

366   This study evaluated a novel next-generation non-fucosylated anti-CTLA4 antibody

367   (BMS-986218) engineered to enhance ADCC in combination with ADT versus ADT

368   alone prior to surgery in men with high-risk localized PCa. We demonstrated that anti-

369   CTLA4-NF shows promising signs of biological activity to counteract ADT-induced Treg

370   expansion in patients, consistent with its proposed mechanism of action, and promotes

371   successful immune priming in PCa. Anti-CTLA4-NF treatment also demonstrated a

372   tolerable safety profile in the pre-surgical setting and potential clinical activity with a

373   lower-than-expected rate of recurrence.

374   To determine potential mechanisms underlying the immunological effects of anti-

375   CTLA4-NF on the TME, we utilized multiple analytic methods to interrogate changes in

376   immune phenotypes at the single-cell level on prostatectomy specimens. These studies

377   revealed both expected and novel associations with response to therapy. We showed

378   for the first time in patients that anti-CTLA4-NF elicits measurable TI-Treg depletion

379   through engagement of the activating FcγR CD16a on tumor macrophages and that the

380   depth of Treg depletion correlates with clinical outcome. Our findings thus support the

381   mechanism established in pre-clinical models but for which limited clinical evidence

382   currently exists[16,29]. These findings also support the hypothesis that first-generation anti-

383   CTLA4 antibodies ipilimumab and tremelimumab fail to deplete TI-Tregs in humans due

384   to inefficient engagement of CD16a on TAMs. A possible explanation for this

385   phenomenon is that non-engineered anti-CTLA4 IgG1 antibodies preferentially engage

386   the inhibitory FcγR *FCGR2B*/CD32b on myeloid cells, preventing TI-Treg-directed

387   ADCC/P[38]. Complicating this mechanism is our observation that inhibitory *FCGR2B* and

18

388   activating *FCGR3A* receptors are highly co-expressed on tumor associated

389   macrophages. Additional studies are warranted to understand how co-expression

390   patterns of *FCGR3A* and *FCGR2B* on TAMs influences efficiency of Treg depletion by

391   Fc-engineered antibodies targeting CTLA-4. An alternative mechanism supported by

392   our data is that tumors with low baseline frequencies of *FCGR3A*/CD16a+ TAMs – or

393   physical segregation of these macrophages from TI-Tregs – may fail to respond to anti-

394   CTLA4-NF. A positive role for *FCGR3A*/CD16a^{+} TAMs in the context of anti-CTLA4-NF

395   is notable, as *FCGR3A*/CD16a^{+} TAMs are generally enriched for the

396   immunosuppressive, M2-like phenotype (e.g. TREM2^{+} and CD206^{+} TAMs in our study)

397   that plays a negative prognostic role in many cancers[22]. However, the role of these

398   TAMs may vary in distinct therapeutic contexts. Recent data suggest M2-like TAMs may

399   in fact be necessary to organize antitumor immune responses[39], which together with our

400   findings challenges the narrative that M2-like TAMs are universally antagonistic to

401   cancer immunotherapies. In either case, pre-clinical studies suggest anti-CTLA4-

402   mediated FcγR engagement on tumor macrophages can elicit pro-inflammatory

403   macrophage skewing[40]. Therefore, Fc-engineered anti-CTLA4 antibodies may deliver a

404   broadly applicable, and possibly synergistic, dual benefit of TI-Treg depletion and M2-

405   to-M1 macrophage skewing that may be effective in overcoming the

406   immunosuppressive microenvironment of many tumor types that do not respond to ICB.

407   Although anti-CTLA4-NF decreased TI-Treg frequency in PCa patients, TI-Treg

408   depletion was not complete. Residual Tregs in tumors following treatment with anti-

409   CTLA4-NF appear to exhibit a highly activated phenotype. While our current studies

410   were not able to confirm whether these Tregs were functionally immunosuppressive,

19

411   these findings indicate a potential limitation of TI-Treg depleting therapies that target a

412   single coinhibitory receptor such as CTLA4. We hypothesize that TI-Tregs undergoing

413   blockade of CTLA4 without experiencing ADCC/P (whether through engagement of

414   *FCGR2B* on proximal TAMs or spatial segregation from ADCC/P effectors) become

415   activated as a result of less coinhibitory receptor activity, as has been observed in mice

416   receiving anti-CTLA4 or in context of anti-PD1 blockade[37,41]. Notably, we found these

417   Tregs to be enriched in other potentially targetable surface proteins, such as CCR8,

418   CD25, CD39, and 4-1BB. Fc competent and glycoengineered antibodies targeting

419   CCR8 are currently in clinical development[42,43]. Thus, co-delivery of glycoengineered

420   antibodies targeting CTLA-4 and CCR8 may be additive or synergistic, given the

421   potential capacity for anti-CCR8 to deplete TI-Tregs remaining following anti-CTLA4,

422   and that anti-CTLA4 elicits enhanced T cell priming and myeloid activation. Recent

423   studies also suggest CD4 Tconv in tumors depleted of TI-Tregs upregulate CCR8 and

424   become functionally immunosuppressive[44]. These studies warrant additional basic and

425   clinical studies of glycoengineered antibodies targeting CTLA4 and CCR8 in prostate or

426   other cancers.

427   In addition to Treg depletion, we found that our glycoengineered anti-CTLA4

428   antibody enhanced T cell priming, potentially by increasing interactions between

429   CTLA4+ effector T cells and FcγR+ dendritic cells. High tumor DC frequencies in our

430   data were significantly associated with lack of disease recurrence in patients receiving

431   ADT + anti-CTLA4-NF. In addition, only patients treated with anti-CTLA4-NF elicited the

432   distinct CD39$^+$41BB$^+$ phenotype in tumor-infiltrating CD8 and CD4 T cells, mirroring

433   established phenotypes for tumor-specific T cells (CD39$^+$CD103^{+35}) and recently primed

20

434   tumor-specific cells (41BB^{+}). Our pre-clinical studies in the murine syngeneic MycCaP

435   model were in striking agreement with these clinical data, and prior *in vitro* work with

436   human T cells and DCs in context of bacterial superantigen-mediated stimulation

437   suggest FcγR engagement on APCs is required for enhanced priming activity of

438   antibodies targeting CTLA-4, independent of Treg depletion[45]. Our pre-clinical findings

439   that FcγR-engaging – but not FcγR-silenced – anti-CTLA-4 antibodies elicit a significant

440   phenotypic shift in tumor DCs suggests a bi-directional effect on both T cells and DCs,

441   reminiscent of observed inflammatory polarization of macrophages post-anti-CTLA-4 in

442   other murine models[40]. Nevertheless, increasing evidence indicates spatial co-

443   localization of effector CD8 and CD4 T cells with DCs in tumors (termed immune

444   "triads," "hubs" or "networks"[46,47]) is critical for productive antitumor immune responses.

445   Further investigation is warranted to determine whether Fc-enhanced antibodies

446   targeting CTLA-4 or other immune checkpoints can facilitate accumulation of these

447   specialized immune clusters in human tumors, and whether this is an essential

448   underlying mechanism of action of these therapeutics.

449   One limitation of our study was the relatively small sample size making

450   interpretation of clinical activity of ADT + anti-CTLA4-NF challenging. Although we

451   observed a lower rate of recurrence following surgery than would be expected based on

452   established risk nomograms, both ADT alone and ADT plus anti-CTLA4-NF

453   demonstrated favorable outcomes. A second limitation was that our study was not

454   designed with sufficient power to compare outcome differences between the treatment

455   arms. In terms of profiling data; given that our CyTOF and scRNAseq was performed on

456   freshly collected specimens, all comparisons were made between treatment groups as

457   opposed to matched pre- and post-treatment tissue, which was not available. However,

458   the arms were relatively well balanced and previous data suggests comparisons

459   between pre-treatment biopsies and surgical resection tissue may be confounded when

460   assessing the immune TME[48]. Clinically, it is notable that development of BMS-986218

461   has been discontinued (NCT03110107), however, several related second-generation

462   depleting anti-CTLA4 antibody agents (botensilimab/AGEN1181, ONC-392, ADG216)

463   are in clinical development. Of note, recent findings from Chand *et al.* indicate

464   botensilimab, an Fc-enhanced DLE-mutated anti-CTLA4-hIgG1 antibody, depletes TI-

465   Tregs in an FcγR-dependent fashion across cancer types and potentially modulates

466   DCs, closely mirroring our pre-clinical and clinical findings with BMS-986218 in PCa[12].

467   Unfortunately, ICB has been ineffective for the vast majority of patients with

468   prostate cancer. However, a more nuanced appreciation of key mechanisms of immune

469   tolerance in prostate cancer and how these evolve with disease progression and vary

470   by site of disease is allowing for the development of novel therapeutic approaches. Our

471   study builds on prior work demonstrating that ADT rapidly elicits a particularly robust

472   immune response in the neoadjuvant setting but is limited by adaptive Treg

473   resistance[11]. In murine prostate cancer models ADT combined with Treg depletion

474   results in durable tumor control supporting the development of novel Treg depletion

475   strategies in patients[10]. Prior studies have evaluated ipilimumab prior to surgery in

476   prostate cancer but have demonstrated limited efficacy and significant toxicity[49] (also

477   see NCT02020070).

478   Although traditional drug development has focused on heavily pre-treated

479   advanced disease first to establish clinical efficacy before moving to earlier disease

22

480 settings, this approach has several shortcomings in the context of immunotherapy. In

481 particular the neoadjuvant setting may be uniquely effective given the abundance of

482 tumor antigens, presence of tumor-draining lymph nodes to allow for tumor antigen

483 cross-presentation and T cell priming, and overall more permissive immune tumor

484 microenvironment. Furthermore, the neoadjuvant setting opens a unique window of

485 opportunity to rapidly assess biological mechanisms of response versus resistance that

486 are challenging in other disease settings. In the NeoRED-P study, we establish the

487 feasibility of such an approach using a novel agent that has not previously been tested

488 in prostate cancer.

489   In conclusion, we demonstrate the safety and feasibility of anti-CTLA4-NF (BMS-

490 986218) in combination with ADT prior to surgery in men with high-risk localized

491 prostate cancer. Our robust immune profiling studies provide first-in-human evidence of

492 TI-Treg depletion by glycoengineered antibodies targeting CTLA-4. In combination with

493 pre-clinical modeling these findings support both canonical and novel mechanisms

494 underlying activity of ADCC/P-competent anti-CTLA4. Our study also identifies

495 immunological correlates of clinical response to ADT + anti-CTLA4-NF, supporting a

496 potential role for immunotherapy targeting CTLA-4 in patients with prostate cancer.

497

23

Tables

Table 1. Patient Baseline Demographics and Disease Characteristics

Characteristic	ADT N=10[1]	ADT + αCTLA-4-NF N=14[1]
Age		
Median (Range)	66.0 (52.0, 72.0)	65.5 (52.0, 77.0)
Race, n (%)		
White	6 (60%)	9 (64%)
Black	2 (20%)	2 (14%)
Asian	1 (10%)	0 (0%)
Other	1 (10%)	3 (21%)
Ethnicity, n (%)		
Not Hispanic	7 (70%)	12 (86%)
Hispanic	3 (30%)	2 (14%)
PSA[2]		
Median (Range)	18.44 (2.37, 94.40)	9.08 (4.57, 364.70)
Testosterone		
Median (Range)	420.30 (153.90, 553.30)	399.50 (273.80, 783.30)
Grade group, n (%)		
3	5 (50%)	2 (14%)
4	3 (30%)	5 (36%)
5	2 (20%)	7 (50%)
T stage, n (%)		
T2	5 (50%)	4 (29%)
T3a	2 (20%)	5 (36%)
T3b	3 (30%)	5 (36%)
Nodal involvement, n (%)	2 (20%)	3 (21%)
Surgical margin involvement, n (%)	3 (30%)	3 (21%)
2-year recurrence-free probability		
Median (Range)	69.50 (8.00, 88.00)	55.00 (4.00, 82.00)
VHR, n (%)[3]	8 (80%)	12 (86%)

[1] Median (Range) for continuous variables; N (%) for categorical variables

[2] Prostate-specific antigen (PSA), ng/mL

[3] Very high-risk (VHR) group defined by National Comprehensive Cancer Network (NCCN) criteria as Gleason ≥8 or PSA >20 ng/ml or clinical T3 stage or higher

Table 2. Treatment-related adverse events

Treatment-related adverse events (TRAEs)[1]	ADT N = 10		ADT + αCTLA-4-NF N = 14	
	All grades	Grade ≥3	All grades	Grade ≥3
Any treatment-related adverse event (TRAE)	8 (80)	—	10 (71)	1 (7.1)
Gastrointestinal disorders	—	—	3 (21)	—
Colitis	—	—	1 (7.1)	—
Diarrhea	—	—	1 (7.1)	—
Elevated transaminases	—	—	1 (7.1)	—
General disorders and administration site conditions	7 (70)	—	8 (57)	—
Chills	1 (10)	—	—	—
Fatigue	—	—	4 (29)	—
Hot flashes	5 (50)	—	6 (43)	—
Injection site reaction	6 (60)	—	7 (50)	—
Investigations	—	—	—	1 (7.1)
Lipase increased	—	—	—	1 (7.1)
Musculoskeletal and connective tissue disorders	—	—	1 (7.1)	—
Bone pain	—	—	1 (7.1)	—
Nervous system disorders	—	—	1 (7.1)	—
Headache	—	—	1 (7.1)	—
Reproductive system disorders	2 (20)	—	—	—
Erectile dysfunction	2 (20)	—	—	—
Skin and subcutaneous tissue disorders	—	—	1 (7.1)	—
Pruritus	—	—	1 (7.1)	—

[1] Treatment-related adverse events (TRAEs) graded according to NCI CTCAE v5.0

Table 3. Study Outcomes

Outcome	N	ADT N=10[1]	ADT + αCTLA-4-NF N=14[1]
PSA response rate[2]	22	3 (33%)	7 (54%)
Undetectable PSA at 12 months	23	8 (80%)	10 (77%)
Time recurrence-free (months)	23		
Median (Range)		23.8 (2.2, 25.3)	22.9 (3.2, 26.6)
Overall recurrence	24	2 (20%)	4 (29%)

[1] Median (Range) for continuous variables; N (%) for categorical variables
[2] PSA reponse rate defined as >50% decrease in PSA prior to prostatectomy

Figure Legends

498 **Figure 1: NeoRED-P trial design and clinical outcomes. (A)** Schematic of NeoRED-

499 P clinical trial design. **(B)** NeoRED-P trial consort diagram. **(C)** Kaplan-Meier curve of

500 PSA recurrence-free survival in patients treated with neoadjuvant ADT. **(D)** Kaplan-

501 Meier curve of PSA recurrence-free survival in patients treated with neoadjuvant ADT +

502 anti-CTLA4-NF. Predicted median 2-year RFS in **(C)** and **(D)** were calculated from the

503 Memorial Sloan Kettering Cancer Center Post-Radical Prostatectomy Prediction risk

504 nomogram. **(E)**. Percent change in serum PSA from pre-treatment to time of surgery in

505 ADT arm patients. Dotted line denotes 50% decline in PSA (PSA50). **(F)** Percent

506 change in serum PSA from pre-treatment to time of surgery in ADT + anti-CTLA4-NF

507 arm patients.

508

509

510 **Figure 2: Multi-omic single cell analysis of Treg depletion by ADT + anti-CTLA4-**

511 **NF. (A)** Schematic of immune correlates performed. **(B)** Summary of data collection and

512 QC passing at a per-patient level for CyTOF, scRNAseq, and immunofluorescence

513 datasets. Patients are additionally annotated according to presence or absence of PSA

514 recurrence within 2 years post-surgery. **(C)** Representative images from whole-slide

515 immunofluorescence data for CD4 and FoxP3 with DAPI nuclear staining. Inset grey

516 box shows a representative CD4$^+$FoxP3$^+$ cell at 2.5x magnification relative to the

517 original image. Scale bar = 40μm. **(D)** Violin plot representing Treg density as measured

518 by Treg cell number per mm^2 between NeoRED-P treatment groups and untreated

519 controls in the immunofluorescence dataset. **(E)** UMAP plot of single cell RNAseq data

520   from all patients, clustered using the PICSES analytical pipeline. **(F)** Bubble plot of

521   representative gene expression for each cluster identified in **(E). (G)** Supervised UMAP

522   plot of CyTOF data from all patients, clustered using FlowSOM. **(H)** Heatmap of lineage-

523   defining protein expression across all clusters. Data represents geometric MFI for each

524   marker normalized to the minimum and maximum values across all clusters. **(I)** UMAP

525   plot representing subclustering of the scRNAseq T/NK cluster shown in **(E)**. **(J)**

526   Visualization of per-cell normalized enrichment scores for a published Treg gene set

527   across all T/NK cells in the scRNAseq dataset. **(K)** Bubble plot of inferred protein

528   activity for manually selected Treg-related proteins across all T/NK subclusters as

529   calculated by VIPER. **(L)** Violin plot representing Treg frequencies as a percent of total

530   cells between NeoRED-P treatment groups and untreated controls in the scRNAseq

531   data. **(M)** Overlay of FlowSOM-derived Treg and CD4 Tconv clusters in the CyTOF data

532   on bi-axial plots representing canonical Treg phenotypes. **(N)** Heatmap of all Treg-

533   relevant phenotypic marker expression in the CyTOF panel, normalized as described in

534   **(H).** Markers with universally high or low expression were scaled according to the

535   minimum and maximum of all FlowSOM clusters. **(O)** Violin plot representing Treg

536   frequencies as a percent of total cells between NeoRED-P treatment groups and

537   untreated controls in the CyTOF data. All statistical tests in this figure utilized Student's t

538   test.

539

540   **Figure 3: Phenotypic analysis of TI-Tregs and association with clinical outcomes.**

541   **(A)** PaCMAP plot representing FlowSOM-derived Treg clusters in the CyTOF data. Data

542   presents 17,757 total cells across 25 patients with evaluable data. Clusters were

27

543   manually annotated according to expression of relevant protein markers as visualized in

544   **(B)** as colorimetric expression maps of selected markers overlayed on the parent

545   PaCMAP plot, or **(C)** heatmap expression of all relevant markers across all clusters.

546   Data represents geometric MFI for each marker normalized to the minimum and

547   maximum values across all clusters. Markers with universally high or low expression

548   were scaled according to the minimum and maximum of all CD45$^+$ FlowSOM clusters.

549   **(D)** Density plots representing Treg cell location on the PaCMAP plot in **(A)** iterated by

550   treatment group. **(E)** Stacked box plot quantifying relative frequencies of all Treg

551   FlowSOM-derived clusters as a percent of Tregs the PaCMAP plot in **(A)** iterated by

552   treatment group. **(F)** Bubble plot representing relative expression of phenotypic markers

553   in Tregs from ADT + anti-CTLA4-NF group versus Tregs in the ADT only group, as

554   calculated by log$_2$ transformed ratio of average geometric MFI of each marker on Tregs

555   from ADT + anti-CTLA4-NF versus ADT groups. Bubble size represents relative gMFI,

556   while color intensity represents percent expression of indicated markers across all

557   Tregs in the dataset. Red color indicates higher relative gMFI in Tregs from patients

558   treated with ADT + anti-CTLA4-NF, while blue color indicates higher relative gMFI in

559   patients treated with ADT only. **(G)** Violin plot representing frequency of Tregs at time of

560   surgery in the scRNAseq dataset stratified by 2-year PSA recurrence status. Student's

561   t-test was performed to evaluate statistical significance. **(H)** Kaplan-Meier curve

562   representing time-to-PSA recurrence in ADT + anti-CTLA4-NF treated patients stratified

563   by Treg frequency (in scRNAseq data). Log-rank test was performed to evaluate

564   statistical significance.

565

566 **Figure 4: Myeloid correlates of Treg depletion and response to anti-CTLA4-NF. (A)**

567 UMAP plot representing subclustering of myeloid cells in the scRNAseq dataset utilizing

568 the PISCES pipeline. **(B)** Visualization of representative gene expression overlayed on

569 the parent UMAP plot to validate cluster annotations. **(C)** Bubble plot validating

570 expression of additional cluster defining genes. **(D)** Visualization of VIPER-inferred

571 protein activity of select Fcγ receptors across myeloid clusters in UMAP space. **(E)**

572 Violin plot of *FCGR3A*/CD16a inferred protein activity on a per cell level stratified by

573 myeloid cluster. **(F)** Correlation between Treg frequency and average inferred protein

574 activity of *FCGR3A*/CD16a across all myeloid cells on a per patient basis, stratified by

575 treatment group. **(G)** Correlation between Treg frequency and average inferred protein

576 activity of *FCGR3A*/CD16a across TREM2$^+$ TAM cluster cells on a per patient basis,

577 stratified by treatment group. Pearson correlations were utilized to evaluate statistical

578 significance of correlations. **(H)** Violin plot of myeloid cluster frequencies as a percent of

579 myeloid cells stratified by treatment group. **(I)** Violin plot of myeloid cluster frequencies

580 as percent of myeloid cells stratified by patient 2-year PSA recurrence status. Student's

581 t-test was utilized to test statistical significance. **(J)** Kaplan-Meier curve representing

582 time-to-PSA recurrence in patients stratified by dendritic cell frequency. Log-rank test

583 was performed to evaluate statistical significance. **(K)** Schematic of pre-clinical

584 validation experiment in the MycCaP model. **(L)** Percent change in tumor volume

585 relative to baseline volume prior to ADT stratified by treatment group. Average values

586 are represented by solid lines and standard deviations are represented by shaded

587 areas. **(M)** Kaplan-Meier curve representing survival of mice shown in **(K-L)**. Log-rank

588 (Mantel-Cox) test was used to evaluate statistical significance. **(N)** Top: Supervised

589   UMAP of live CD45$^+$ cells from MycCaP tumors harvested following therapy as denoted

590   in **(K-M)**. Semi-supervised clustering was performed using FlowSOM and clusters were

591   manually annotated according to expression of lineage-defining markers. Bottom:

592   Heatmap of lineage-defining markers across all clusters. Data represents geometric MFI

593   for each marker normalized to the minimum and maximum values across all clusters.

594   **(O)** Frequency of TI-Tregs and cDCs as percent of all live CD45$^+$ cells, stratified by

595   treatment group. **(P)** PaCMAP representing FlowSOM-derived cDC clusters. Clusters

596   were manually annotated according to expression of canonical marker proteins,

597   representative markers shown at right. Below: Pseudocolor representation of cDC

598   phenotypes in PaCMAP space stratified by treatment group. **(Q)** Violin plot representing

599   frequency of PD-L2$^+$ DCs as percent of all live CD45+ events stratified by treatment

600   group. Student's t-test was used to assess statistical significance. All murine data

601   shown is representative of two independent experiments of 5-10 mice per group each

602   for survival and immune profiling studies.

603

604   **Figure 5: Phenotypic modulation and induced priming of CD8 T cells by anti-**

605   **CTLA4-NF. (A)** UMAP plot representing subclustering of CD8 T cells in the scRNAseq

606   data utilizing the PICSES pipeline. **(B)** Visualization of representative gene expression

607   signatures or inferred activity of indicated proteins overlayed on the parent UMAP plot to

608   validate cluster annotations **(C)** Violin plot of CD8 T cell cluster frequencies as a percent

609   of CD8 T cells stratified by treatment group. **(D)** PaCMAP plot of NeoRED-P patient

610   tumor-infiltrating CD8 T cells by CyTOF. Clusters were derived from FlowSOM. **(E)**

611   Pseudocolor density plots of CD8 T cells in PaCMAP space stratified by treatment

612 group. **(F)** Expression of CD39 and 4-1BB by geometric MFI on CD8 T cells as

613 represented by color mapping on PaCMAP plot. **(G)** Violin plot representing frequency

614 of manually gated CD39$^+$4-1BB$^+$ CD8 T cells as a percentage of all CD8 T cells

615 stratified by treatment group. **(H)** PaCMAP plot of NeoRED-P patient tumor-infiltrating

616 CD4$^+$ FoxP3$^-$ Tconv cells by CyTOF. Clusters were derived from FlowSOM. **(I)**

617 Pseudocolor density plots of CD4 Tconv cells in PaCMAP space stratified by treatment

618 group. **(J)** Expression of CD39 and 4-1BB by geometric MFI on CD4 Tconv as

619 represented by color mapping on PaCMAP plot. **(K)** Violin plot representing frequency

620 of manually gated CD39$^+$4-1BB$^+$ CD4 Tconv cells as a percentage of all CD8 T cells

621 stratified by treatment group. **(L)** PaCMAP plot of MycCaP-infiltrating CD8 T cells by 45-

622 parameter flow cytometry in response to ADT, ADT + anti-CTLA4 (ND), or ADT + anti-

623 CTLA4 (D). Clusters were derived from FlowSOM. **(M)** Pseudocolor density plots of

624 CD8 T cells in PaCMAP space stratified by treatment group. **(N)** Expression of CD39

625 and 4-1BB by geometric MFI on CD8 T cells as represented by color mapping on

626 PaCMAP plot. **(O)** Violin plot representing frequency of manually gated CD39$^+$4-1BB$^+$

627 CD8 T cells as a percentage of all CD8 T cells stratified by treatment group. **(P)** Biaxial

628 plots representing expression of CD44 and Ki67 on CD8 T cells in tumor draining lymph

629 nodes of mice shown in **(L-O)**. **(Q)** Biaxial plots representing expression of CD44 and

630 Ki67 on CD4$^+$FoxP3$^-$ Tconv cells in tumor draining lymph nodes of mice shown in **(L-O)**.

631 **(R)** Violin plots representing frequencies of CD44$^+$Ki67$^+$ CD8 and CD4 Tconv cells as a

632 percentage of parent populations stratified by treatment group. Student's t-test was

633 used to assess statistical significance. All murine data shown is representative of two

31

634   independent experiments of 5-10 mice per group each for survival and immune profiling

635   studies.

636

Methods

Patients

Key eligibility criteria included histologically confirmed adenocarcinoma of the prostate (clinical stage T1c-T3b, N0, M0) with no detectable metastatic involvement of lymph nodes, bone, or visceral organs and high-risk disease as defined by central pathologic review of at least two biopsy cores with Gleason sums of ≥4+3. Patients were required to demonstrate adequate bone marrow, hepatic, and renal function. Prior exposure to radiation therapy, chemotherapy, immunotherapy, biologic therapy, or concomitant treatment with hormonal therapy and/or systemic corticosteroids (with the exception of short courses <5 days 7 days or more prior to treatment initiation) was not allowed. See full trial protocol in Supplementary Information for additional detail.

Clinical Trial Regulatory Oversight and Approval

This trial was approved and all amendments reviewed by the Columbia University Institutional Review Board (IRB) under the protocol number AAAS3560. The trial was registered with ClinicalTrials.gov on March 6, 2020, with the ClinicalTrials.gov ID NCT04301414. All patients provided informed consent prior to enrollment in the study.

Clinical Trial Design

This was a single-center, randomized, two-arm, open label pilot study conducted at Columbia University Irving Medical Center to evaluate the safety, feasibility, and immunogenicity of neoadjuvant degarelix (ADT) or BMS-986218 (anti-CTLA4-NF) plus ADT prior to radical prostatectomy in men with high-risk localized prostate cancer.24 patients were enrolled in the study. The first 4 patients were assigned to receive ADT + anti-CTLA4-NF as a safety lead-in. Thereafter, 10 patients each were randomized to the two treatment arms. In the ADT-only arm, men received 240 mg degarelix acetate subcutaneously two weeks prior to surgery. In the ADT + anti-CTLA4-NF arm, men received 20 mg BMS-986218 intravenously on day 1 and day 15, and 240 mg degarelix acetate subcutaneously on day 8, starting 3 weeks prior to prostatectomy (Figure 1A).

Clinical Trial Endpoints and Assessments

666   The primary objective was to characterize safety, tolerability, and feasibility of ADT with

667   or without anti-CTLA4-NF in the neoadjuvant setting. The secondary objectives

668   included: (i) evaluation an immune response consistent with the proposed mechanism

669   of action of anti-CTLA4-NF, specifically the effect on intratumoral Treg density and

670   CD8:Treg ratio, (ii) estimation of the pathologic complete response rate following

671   neoadjuvant anti-CTLA4-NF plus ADT as compared to ADT alone, (iii) estimation of the

672   PSA50 response rate and rate of undetectable PSA at 12 months following neoadjuvant

673   anti-CTLA4-NF plus ADT as compared to ADT alone, and (iv) estimation of the rate of

674   post-surgical PSA recurrence within two years of completing therapy between patients

675   receiving ADT alone versus ADT plus anti-CTLA4-NF.

676   Serum PSA and testosterone levels were measured on all patients prior to therapy

677   initiation and at time of surgery. Thereafter, serum PSA and testosterone was measured

678   on all patients every 3 (±1) months during the first post-operative year and every 6 (±2)

679   months during the second and third post-operative years. Adverse events were

680   monitored during the course of therapy and until at least two years post-surgery and

681   registered according to CTCAE version 5.0. Adverse events were deemed associated

682   or not associated with ADT and/or anti-CTLA4-NF according to the treating physician.

683   Peripheral blood was drawn weekly during the course of treatment and at follow-up

684   visits after surgery. Tumor samples from prostatectomy specimens were collected at

685   time of surgery; fresh tissue was collected and processed as described below, and

686   archival formal-fixed paraffin-embedded (FFPE) tissues were obtained for indicated

687   correlative studies.

688   **Tumor Sample Collection & Processing**

689   Following radical prostatectomy, surgical tissues were grossed and sectioned by

690   pathologists at Columbia University Irving Medical Center. Up to 1 gram of tumor tissue

691   was collected in Miltenyi MACS Tissue Storage Solution on wet ice, massed, washed

692   with PBS, then diced with microdissection scissors to 1-2mm pieces in a 5mL

693   Eppendorf tube. Tissue pieces were transferred into a Miltenyi C tube (0.5g per tube) in

694   serum free RPMI, then enzymes H, R, and A were added according to the Miltenyi

695   Human Tumor Dissociation Kit protocol. Tumors were digested on a gentleMACS

696   Octodissociator under program 37_multi_A_01. Once finished, RPMI supplemented

697   with 5% FBS (R5 media) was added to quench digest enzymes, then suspensions were

698   filtered (70um) into a 15mL conical tube and spun at 400xg for 5 minutes at 4° C.

699   Pellets were RBC lysed by resuspension in Miltenyi 1x ACK buffer for 5 minutes at RT,

700   followed by centrifugation at 300xg for 5 min at 4° C. Pellets were washed with R5,

701   filtered, and pelleted. Cell numbers were then quantified on a Countess II automated

702   cell counter, then samples were split for downstream analysis by CyTOF and single cell

703   RNA sequencing.

704   **Tumor Whole Exome Sequencing**

705   Baseline germline mutation testing was performed at trial screening using the Invitae

706   Common Hereditary Cancers Panel (48 genes) or the Invitae Multi-Cancer Panel (70

707   genes). Archival formalin-fixed, paraffin-embedded (FFPE) tumor specimens with >30-

708   40% tumor content were sectioned at 5uM by the Herbert Irving Comprehensive Cancer

709   Center's (HICCC) Molecular Pathology Shared Resource (MPSR). Hematoxylin and

710   eosin (H&E) slides were reviewed and tumor area marked by a board-certified

711   pathologist. Tissue sections were submitted for mutational profiling to Columbia

712   University Irving Medical Center's Laboratory of Personalized Genomic Medicine, a

713   CAP-accredited and CLIA-certified clinical genomics laboratory. Tissue was prepared

714   for sequencing as previously described[50].

715   Targeted next-generation sequencing of tumor DNA and cDNA was performed using

716   the Columbia Combined Cancer Panel (CCCP), a New York State Department of

717   Health-certified panel, covering 467 cancer-related genes Raw sequencing data were

718   mapped to the human reference genome (GRCh37, hg19), and analyzed for single

719   nucleotide variants, small insertions and deletions, copy number alterations, gene

720   rearrangements and fusions, tumor mutation burden, and microsatellite instability using

721   validated clinical pipelines. Identified genomic alterations were reviewed and interpreted

722   for pathogenicity and clinical significance by a board-certified molecular pathologist. See

723   supplementary Information for genes covered in all genomic testing panels and panel-

724   specific methodologies.

725

Mass Cytometry

Cell pellets were resuspended in R5 supplemented with Rh103 viability stain (1:500) in a 1.5mL protein lo-bind tube and incubated 15 min at 37° C. Cells were pelleted, washed once with R5 media, then resuspended in 49ul Cell Staining Buffer (CSB; Fluidigm) followed by addition of 1ul Fc Block (TruStain FcX; BioLegend). Samples were mixed and incubated for 5-10 minutes on ice. Separate surface and intracellular antibody cocktails were pre-mixed and stored at -80° C; during the Fc Block incubation cocktails were thawed on ice, the surface mix was spun through a PVDF centrifuge filter for 3 min at 1200rpm, then 50ul surface mix was added to the cell suspension for a final volume of 100ul. Surface staining was conducted on ice for 30 minutes, then cells were washed twice with 1mL CSB. Cells were fixed by resuspending in 500ul FoxP3 Fixation/Permeabilization buffer (eBioscience) and incubating at RT for 30 minutes. Fixative was washed out with two successive washing steps with 1mL 1X Perm Wash. For intracellular staining, cells were resuspended in 49ul 1X PW + 1ul heparin on ice. Intracellular cocktails were diluted to 50ul with 1X PW and were spun through a PVDF membrane prior to addition to the cell solution for a final staining volume of 100ul. Cell staining was conducted on ice for 30 minutes, followed by two successive washes with 1mL 1X PW. Fully stained cells were fixed in 400ul FixIR solution (2.4% PFA + 0.25 nM Ir-191/193) at room temperature for 30 minutes. Samples were washed with 600ul CSB and spun for 4 min at 600 xg, and washed again with 1mL CSB. Samples were either resuspended in CSB and held at 4° C for a maximum of 72 hours before being acquired on a CyTOF Helios at the Columbia University Human Immune Monitoring Core (HIMC), or frozen at -80° C in FBS + 10% DMSO before being thawed and acquired on a CyTOF Helios at either the Columbia University HIMC or the Mayo Clinic Immune Monitoring Core (IMC). All samples were acquired with, and data was normalized using spiked in four-element EQ Calibration Beads (Fluidigm/Standard BioTools) for longitudinal and cross-platform standardization.

Single Cell RNA Sequencing and Analysis

Cells were processed for scRNAseq using the 10X Chromium 3' v2 Library and Gel Bead Kit (10x Genomics) according to manufacturer's instructions at the Columbia

36

756   University HIMC and as described previously[22]. ScRNASeq data were processed with

757   Cell Ranger software at the Columbia University Single Cell Analysis Core. Illumina

758   base call files were converted to FASTQ files with the command "cellranger mkfastq."

759   Expression data were processed with "cellranger count" on the pre-built human

760   reference set of 30,727 genes. Cell Ranger performed default filtering for quality control.

761   The Seurat package was used for quality control filtering of cells, excluding those with

762   fewer than 10% mitochondrial RNA content, more than 1,500 unique UMI counts, and

763   fewer than 15,000 unique UMI counts. Pooled distribution across all samples of UMI

764   counts, unique gene counts, and percentage of mitochondrial DNA after QC-filtering is

765   shown in Extended Data Figure 3A.

766   *Gene Expression Clustering and Initial Cell Type Inference |* Combined and normalized

767   gene expression matrix was visualized by UMAP projection and unsupervised clustering

768   was performed using the Louvain algorithm applied to top50 principal components of

769   the full data matrix, with optimal clustering resolution determined by bootstrapped

770   silhouette score optimization, as described in prior work[22]. Broad cell types were

771   inferred by SingleR with blueprint-ENCODE reference of sorted cell types, with each

772   cluster consensus-labelled by majority-inferred cell type[24]. Further re-labelling of tumor

773   cell clusters (labelled as epithelial by SingleR) was performed by InferCNV analysis,

774   demonstrating consistent regions of chromosomal aberration above background rate

775   seen in other cell types[26].

776   *Regulatory Network Inference |* For each gene expression cluster, a gene regulatory

777   network was inferred on log-normalized counts by the ARACNe algorithm[51] to identify

778   downstream transcriptional targets of regulatory proteins. ARACNe was run with 100

779   bootstrap iterations using 1785 transcription factors (genes annotated in gene ontology

780   molecular function database as GO:0003700, "transcription factor activity," or as

781   GO:0003677, "DNA binding" and GO:0030528, "transcription regulator activity," or as

782   GO:0003677 and GO:0045449, "regulation of transcription"), 668 transcriptional

783   cofactors (a manually curated list, not overlapping with the transcription factor list, built

784   upon genes annotated as GO:0003712, "transcription cofactor activity," or GO:0030528

785   or GO:0045449), 3455 signaling pathway related genes (annotated in GO biological

786   process database as GO:0007165, "signal transduction" and in GO cellular component

37

787   database as GO:0005622, "intracellular" or GO:0005886, "plasma membrane"), and

788   3620 surface markers (annotated as GO:0005886 or as GO:0009986, "cell surface").

789   ARACNe is only run on these gene sets so as to limit protein activity inference to

790   proteins with biologically meaningful downstream regulatory targets, and we do not

791   apply ARACNe to infer regulatory networks for proteins with no known signaling or

792   transcriptional activity for which protein activity may be difficult to biologically interpret.

793   Parameters were set to zero DPI (Data Processing Inequality) tolerance and MI (Mutual

794   Information) p value threshold of 10–8, computed by permuting the original dataset as a

795   null model.

796   *Protein Activity Inference and Clustering |* Inference of protein activity was performed

797   from scaled integrated gene expression matrix and the set of all ARACNe-inferred gene

798   regulatory networks using the VIPER algorithm[20,22]. Normalized Enrichment Scores of

799   protein activity were successfully inferred on a per-cell basis for 3,437 regulatory

800   proteins. This protein activity matrix was re-clustered in Seurat using the Louvain

801   algorithm on top50 principal components and clustering resolution parameter optimized

802   by silhouette score, as above, with two-dimensional data projection visualized by

803   UMAP. Differential protein activity between clusters was computed by Wilcoxon rank-

804   sum test with multiple testing correction. Differential abundance of each cluster per

805   patient by treatment group was also assessed by Wilcoxon-rank-sum test, as well as

806   differential abundance of each cluster within the combination-treatment group between

807   patients with and without disease recurrence. Cell clusters inferred by SingleR as

808   lymphoid lineage, myeloid lineage, or tumor cells, respectively, were each isolated for

809   further sub-clustering analyses.

810   *Single-cell RNA-seq T/NK Subclustering |* Subsetting lymphoid cells from initial analysis

811   resulted in a VIPER matrix of 14,208 cells, projected into their first 50 principal

812   components using Seurat's RunPCA function, followed by using Seurat's RunUMAP

813   function with method umap-learn. Unsupervised clustering of lymphoid cells was

814   performed by the same silhouette score optimized Louvain approach as above. In

815   addition to SingleR cell type labelling, we applied GSEA on cell-by-cell basis using an

816   expression-based T-reg marker gene set previously published from Obradovic et. al.

817   Cell 2021[22], to identify the T-reg cluster. VIPER activity of manually selected relevant

818 markers was also visualized for lymphoid cell subtype identification of all other clusters.

819 Differential abundance of each cluster as percent of lymphoid cells was assessed by

820 treatment group and by recurrence vs non-recurrence within the combination-treatment

821 group, by Wilcox rank-sum test. The ADT-only treatment group was not stratified due to

822 insufficient recurrences for statistical power and recurrence data were not available in

823 the untreated group. Further testing in the combination treatment group of association

824 between Treg abundance and time-to-recurrence was performed by Kaplan-Meier

825 analysis, with optimal cut-point of high-Treg vs low-Treg samples determined by log-

826 rank maximization, and p-value assessed by Cox regression.

827 *Single-cell RNA-seq Myeloid Cell Subclustering |* Isolation of myeloid cells (clusters

828 identified by SingleR as predominantly Monocyte or Macrophage) yielded 5771 Myeloid

829 cells, subclustered in the same manner as lymphoid cells above. RunPCA and

830 RunUMAP from Seurat were used to compute the first 50 principal components and

831 UMAP respectively. Upon sub-clustering, a subpopulation identified as fibroblasts

832 (n=183) by SingleR with the BlueprintEncode reference dataset from Celldex was

833 removed from downstream analysis. A 7-cluster unsupervised clustering solution was

834 selected as optimal by maximization of silhouette score. Clusters were then annotated

835 by cell type with a combination of visualizing manually selected phenotypic markers and

836 assessing Gene Set Enrichment of differentially active proteins by cluster.

837 *Correlation Between FCGR3A Activity and Treg Frequency |* In order to assess for

838 relationship between FCGR3A protein activity among myeloid cells and Treg frequency,

839 we performed correlative analysis between Treg frequency as a percent of lymphoid

840 cells for each patient against average FCGR3A protein activity for myeloid cells as a

841 whole in the same patient, as well as for the specific subset of TREM2+ TAMs.

842 Stratifying these analyses by treatment group shows treatment-dependent association

843 between FCGR3A activity and Treg abundance, such that in combination-treatment

844 alone higher myeloid FCGR3A activity was found to associate with lower Treg

845 abundance.

846 *Single-cell RNA-seq CD8+ T Cell Subclustering |* Upon sub-clustering lymphoid cells,

847 we further isolated the two identified clusters of CD8 T-cells (CD8-1 and CD8-2) for

848 deeper sub-phenotyping. This yielded a VIPER protein activity matrix for 7,475 cells.

849   Re-clustering on the top50 principal components of this matrix yielded a four-cluster

850   solution such that one small subset of n=187 cells appeared to be naïve CD8s or

851   potential contamination, and were removed. This conclusion was supported by GSEA

852   on the gene expression signatures of these cells with the Naive CD8 gene set from

853   Zheng et al. 2021[34]. The remaining true CD8+ cells were re-scaled and re-clustered

854   yielding a three-cluster solution. These clusters were labelled as "Central Memory",

855   "Effector" and "Effector/Resident Memory" by Gene Set Enrichment Analysis. "Memory

856   vs Effector" and "Effector vs Memory" signatures were generated using a Mann-Whitney

857   U-Test between the gene expression profiles of CD8+ Trm and CD8+ cytotoxic

858   populations reported by Tuong et al. 2021[31]. The top genes from each of these

859   signatures and in the signatures from Zheng et al. 2021[34] were supplied as gene sets to

860   GSEA to compute enrichment on the gene expression profiles of our CD8+ cells.

861   Relevant manually curated marker genes were further visualized by VIPER activity per

862   cluster. Cluster frequencies by treatment group were then calculated to identify changes

863   in these CD8+ subtypes in response to treatment.

864   **Immunofluorescence**

865   Archival FFPE prostate tumor specimens were selected by a board-certified pathologist.

866   Immunoblank slides and representative adjacent H&E slides were sectioned at 5uM by

867   the Herbert Irving Comprehensive Cancer Center's Molecular Pathology Shared

868   Research (HICCC MPSR). Tissue sections were deparaffinized using a standard series

869   of xylene and ethanol washes and rinsed with distilled water. Antigen retrieval was

870   performed by heating the slides in citrate buffer at pH of 6.0 for 20 minutes in a pressure

871   cooker. Slides were incubated overnight at 4°C with primary antibodies against CD4

872   (1:200, Abcam) and FOXP3 (1:100, Invitrogen). Subsequently, the slides were

873   incubated for 30 minutes at room temperature with horse anti-mouse antibody (1:200,

874   Vector Laboratories), and then for 30 minutes with Streptavidin, Alexa Fluor™ 594

875   conjugate (1:1000, Invitrogen). Next, the slides were incubated for 30 minutes at room

876   temperature with goat anti-rabbit antibody (1:200, Vector Laboratories) and then for an

877   additional 30 minutes with Streptavidin, Alexa Fluor™ 488 conjugate (1:300, Invitrogen).

878   Slide sections were mounted at room temperature with 25uL of VECTASHIELD

879   Vibrance® Antifade Mounting Medium with DAPI (Vector Laboratories) and

880   coverslipped.

881   Fluorescent images were acquired by the PhenoImager™ HT Instrument (Akoya

882   Biosciences). Onboard spectral unmixing was performed using the PhenoImager HT 2.0

883   software (Akoya Biosciences). The images were analyzed with QuPath version 0.5.1[52].

884   Regions of interest were generated referencing tumor-marked H&E slides from adjacent

885   sections. Nuclear and cell segmentation were performed using the QuPath extension

886   StarDist 0.4.0 with the pretrained model dsb2018_heavy_augment.pb[53]. Following

887   cellular segmentation, object classifiers were trained on each image to quantify Tregs

888   (CD4+/FOXP3+) per mm^2 or as a percentage of all DAPI+ cells.

889   **Mice**

890   Male FVB mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were

891   8-10 weeks old at time of use. All animals were housed in strict accordance with NIH

892   and American Association of Laboratory Animal Care regulations. All experiments and

893   procedures for this study were approved by the Mayo Clinic Institutional Animal Care

894   and Use Committee (IACUC).

895   **Animal Studies**

896   Myc-CaP cells were provided as a kind gift from Dr. Charles Drake and were verified as

897   free of pathogen and cell line contamination using a 19-marker mouse STR panel

898   through IDEXX BioAnalytics (Columbia, MO). Myc-CaP cells were passaged in RPMI

899   medium (Corning; Corning, NY) supplemented with 10% FBS (Corning; Corning, NY),

900   100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco; Gaithersburg, MD). For tumor

901   implantation, Myc-CaP cells at 70-90% confluence were harvested with 0.05% trypsin

902   (Corning; Corning, NY), washed with PBS, counted, and resuspended at $10x10^6$

903   cells/mL in ice cold PBS. On day 0, 8–10-week-old mice were implanted on the right

904   flank with $1x10^6$ Myc-CaP cells. Cages were randomized to treatment groups after

905   cages were manually normalized to reduce intergroup variability, using the final tumor

906   volume measurement values available prior to treatment. Tumor measurements were

907   recorded in X, Y, and Z dimensions in a non-blinded fashion (largest diameter, smallest

908   diameter, and largest height, respectively) every 2-3 days by digital caliper and tumor

909   volume was calculated by multiplying X*Y*Z. On day 24 when tumors were

910   approximately 200mm^3 on average, mice received a single subcutaneous injection in

911   the neck scruff of 625 ug degarelix acetate (ADT; Cayman Chemical; Ann Arbor, MI)

912   resuspended in 100 µl sterile water per mouse. Appropriate treatment groups

913   additionally received anti-CTLA4 (D) (9D9-mIgG2a) or anti-CTLA4 (ND) (9D9-mIgG2a-

914   LALAPG) as indicated by intraperitoneal (IP) injection at 100 µg per mouse in 100 µl

915   sterile PBS on days 24, 27, and 30. Antibodies were purchased from BioXCell

916   (Lebanon, NH). For flow cytometry analysis, cohorts of animals were euthanized on day

917   32 for tissue collection and processing. For survival analysis, mice were deemed eligible

918   for euthanasia when tumor volume exceeded 1,000 mm^3 or tumor ulceration exceeded

919   5 mm in diameter.

920   **Spectral Flow Cytometry**

921   *Tissue harvest and dissociation* | Following mouse euthanasia, tumor-draining inguinal

922   lymph nodes were dissected and placed in 48-well plates containing 150 µl R10 media

923   on ice. Tumors were harvested, massed, and up to 50 mg tumor was diced and placed

924   in X-Vivo 15 media (Lonza; Basel, Switzerland) in 15mL Eppendorf tubes on ice. DNase

925   (40 µl of 20 mg/mL solution; Roche; Basel, Switzerland) and Collagenase D (125 µl of

926   40 mg/mL solution; Roche; Basel, Switzerland) were added to tumor samples prior to

927   incubation on a shaker at 37° C for 30 minutes. Digest reactions were quenched by

928   adding 5mL R10 media. Tumor digests were filtered through 70 µm filters (Miltenyi;

929   Bergisch Gladbach, GE), remaining undigested tissue was mashed with the handle end

930   of a 3mL syringe plunger, then filters were washed with 5mL R10 prior to centrifugation.

931   Lymph nodes were physically disaggregated with a 1mL syringe plunger in the 48-well

932   plate. All samples were then transferred in R10 into U-bottom 96-well plates for staining.

933   *Staining protocol* | Samples were washed with PBS. Dead cells were stained by

934   resuspension in 100 µl PBS + Live/Dead Fixable Blue dye (1:500; Invitrogen; Waltham,

935   MA). This and all further staining or fixation steps were performed for 30 minutes at

936   room temperature on a plate shaker protected from light, unless specified otherwise.

937   Samples were washed 2x with PBS, then were resuspended in FACS (PBS + 3% FBS

938   + 1mM EDTA + 10mM HEPES) supplemented with TruStain FcX (1:50; BioLegend; San

939   Diego, CA) and TruStain Monocyte Blocker (1:20; BioLegend; San Diego, CA) and

940   placed on ice. Surface antibodies were prepared at optimal dilutions (see

941   Supplementary Table S1) in FACS supplemented with Brilliant Stain Plus buffer (BD;

942   Franklin Lakes, NJ), then were added to samples in blocking solution. After staining,

943   samples were washed 2x with FACS buffer and fixed in 100 μl of FoxP3

944   Fixation/Permeabilization Kit buffer (eBioscience; San Diego, CA). Samples were

945   washed twice with 1X Permeabilization buffer (1X PW) then were stained in 1X PW plus

946   intracellular antibodies. Plate(s) were sealed and intracellular staining was performed

947   overnight at 4° C on an orbital shaker. Samples were then washed twice in FACS then

948   resuspended in 200 μl FACS and were immediately acquired on a Cytek Aurora 5-laser

949   (UV/V/B/YG/R) cytometer. Simultaneously stained splenocyte samples or SpectraComp

950   beads (Slingshot Biosciences; Emeryville, CA) were utilized for single stain controls as

951   indicated in Supplemental Table S1.

952   **Analysis of Mass Cytometry and Flow Cytometry Data**

953   All analysis was performed using FlowJo (BD; San Diego, CA). Live, single cell, CD45+

954   events from all files were identified by manual gating using conventional gating

955   strategies for CyTOF and flow cytometry data as shown in Extended Data Fig 5-6.

956   Samples with fewer than 1,000 live CD45+ single cell events were excluded from further

957   analysis. All live CD45+ single cell events in the CyTOF data were concatenated for

958   high dimensional analyses, while up to 50,000 live CD45+ single cell events from the

959   flow cytometry dataset were downsampled and concatenated for downstream analysis.

960   Canonical immune lineages in both datasets were defined in a semi-supervised manner

961   utilizing FlowSOM[27]. Data were purposely overclustered to 30 FlowSOM-derived

962   clusters based on expression of lineage-defining markers in each panel. These clusters

963   were then manually collated based on lineage marker expression to obtain the minimal

964   possible number of canonical immune lineages. Clusters were embedded in two-

965   dimensional space for visualization using the supervised UMAP function in FlowJo,

966   which performs dimensionality reduction based on selected lineage-defining marker

967   expression with incorporation of FlowSOM-derived clusters as a weighted parameter.

968   For discovery-based subclustering of individual lineages (e.g. CD4 Tregs), an additional

969   round of semi-supervised FlowSOM was performed in an analogous manner as

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970   described above; involving FlowSOM-derived overclustering based on phenotypic

971   markers differentially expressed by cells within the lineage of interest, followed by

972   manual collation of clusters into minimal number of phenotypically distinct subclusters.

973   For visualization of discovery-based subclustering within lineages, we utilized

974   PaCMAP[54] dimensionality reduction to embed data in two dimensions. Absolute cell

975   numbers and mean geometric fluorescent intensity (gMFI) data was exported for each

976   cluster for downstream analysis. Phenotypic marker data was scaled based on the

977   minimum and maximum gMFI values of each given marker across all live CD45+ cells

978   or within the lineage of interest, as indicated in the figure legends.

979   **Statistical Analyses**

980   Baseline patient characteristics and study outcomes were computed and visualized

981   utilizing the gtsummary package (v 1.7.2)[55], in Rstudio (R version 4.3.1, 2023-06-16).

982   Mutational profiles were visualized using Oviz-Bio's LandScape (v1.1.1)[56]. Stratification

983   for Kaplain Meier analyses was performed using log-rank maximization algorithm as

984   implemented in the survminer package. CyTOF and spectral flow cytometry statistical

985   testing was conducted in GraphPad Prism (v10.2). All other quantitative and statistical

986   analyses were performed using the R computational environment and packages

987   described above. Differential gene expression was assessed at the single-cell level by

988   the MAST single-cell statistical framework as implemented in Seurat v4[57], and

989   differential VIPER activity was assessed by Wilcoxon rank-sum test, each with

990   Benjamini-Hochberg multiple-testing correction. Unless otherwise noted, comparisons

991   of cell frequencies by treatment group were performed by Student's t-test or non-

992   parametric Wilcox rank-sum test, and survival analyses were performed by log-rank

993   test. In all cases, statistical significance was defined as an adjusted p value less than

994   0.05. Details of all statistical tests used can be found in the corresponding figure

995   legends.

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998 **<u>Acknowledgments</u>**

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1007 **Extended Data**

1008

1009 **Extended Data Figure 1: NeoRED-P study co-mutation plot. (A)** Co-mutation plot of

1010 NeoRED-P patients and stage matched untreated control cohort.

1011

1012 **Extended Data Figure 2: PSA responses and testosterone recovery of all trial**

1013 **participants. (A)** Median serum PSA (dark blue line; round dots) and testosterone (light

1014 blue line; squares) concentrations over time of all patients treated with ADT only. Error

1015 bars denote interquartile range. **(B)** Median serum PSA (dark red line; round dots) and

1016 testosterone (light red line; squares) concentrations of all patients treated with ADT +

1017 anti-CTLA4-NF. Error bars denote interquartile range. **(C)** Plots of serum PSA and

1018 testosterone concentrations for individual patients treated with ADT only. **(D)** Plots of

1019 serum PSA and testosterone concentrations for individual patients treated with ADT +

1020 anti-CTLA4-NF. D = day; P or RP = radical prostatectomy; M or Mo = month.

1021

1022 **Extended Data Figure 3: Single cell RNA sequencing QC and inferCNV results. (A)**

1023 Violin plots representing quality control plots of scRNAseq data stratified by treatment

1024 group, including number of unique molecular identifiers (UMIs) per cell (left), number of

1025 unique genes detected per cell (middle), and percent mitochondrial gene content per

1026 cell (right). **(B)** Plots representing outcomes of InferCNV analysis through visualization

1027 of inferred copy number aberrations. Reference immune and stromal cell populations

1028 shown above, and clusters annotated as tumor cells shown at bottom.

1029

1030 **Extended Data Figure 4: Comparison of single cell RNA sequencing and CyTOF**

1031 **datasets. (A)** Representative gating strategy representing isolation of viable, single cell

1032 CD45+ cells in the CyTOF dataset. **(B)** Pearson correlation plots comparing frequency

1033 of immune lineages annotated during clustering of both CyTOF and scRNAseq datasets

1034 as a percent of total cells. **(C)** Violin plots representing frequency and distribution of all

1035 lineage frequencies as percent of total cells by scRNAseq, stratified by treatment group.

1036 **(D)** Violin plots representing frequency and distribution of all lineage frequencies as

1037 percent of total cells by CyTOF, stratified by treatment group. **(E)** Violin plot

1038 representing frequency of T/NK subclusters as a percent of all cells, stratified by post-

1039 surgical 2-year recurrence status. Student's t-test was used to evaluate statistical

1040 significance. **(F)** Kaplan-Meier curve representing time-to-PSA recurrence in ADT-

1041 treated patients stratified by Treg frequency in scRNAseq data. Log-rank test was

1042 performed to evaluate statistical significance.

1043

1044 **Extended Data Figure 5: Additional flow cytometry data related to DC modulation**

1045 **by ADT + anti-CTLA4 (D) in the MycCaP model. (A)** Representative gating strategy of

1046 viable, single cell CD45+ cells in the spectral flow cytometry dataset derived from **(A)**

1047 tumor and **(B)** tumor draining lymph nodes. Summary of downstream analytical

1048 workflow shown at right. **(C)** Heatmap of relevant phenotypic marker expression across

1049 all tumor-infiltrating DC clusters. Data represents median MFI for each marker

1050 normalized to the minimum and maximum values across all DC clusters. Any markers

1051 exhibiting uniform low expression were normalized to the minimum and maximum

1052 values across all immune clusters to demonstrate low expression levels across clusters.

1053   **(D)** Violin plots representing relative proportions of DC clusters as a percentage of all

1054   DCs in the indicated treatment groups. Two-way ANOVA with Tukey multiple testing

1055   correction was used to assess statistical significance. **(E)** Violin plots representing the

1056   frequency of PD-L2+ DCs as a percentage of DCs (left) or as a percentage of CD45

1057   (right) in a second independent replicate experiment. Student's t-test was used to

1058   assess statistical significance.

1059

1060   **Extended Data Figure 6: Additional CD8 phenotyping data by scRNAseq, CyTOF,**

1061   **and flow cytometry. (A)** UMAP plot representing subclustering of combined CD8-1 and

1062   CD8-2 clusters in the original T/NK cluster, identifying 4 phenotypically unique

1063   subclusters. **(B)** Violin plots representing expression of *Cd8a* and *Cd4*, VIPER inferred

1064   protein activity of *CCR7*, and tumor-infiltrating naïve CD8 T cell GSEA scores (derived

1065   from Zheng *et al*. 2021) on a per cell basis in the scRNAseq dataset, stratified by

1066   subcluster. **(C)** Heatmap of relevant phenotypic marker expression across all tumor-

1067   infiltrating CD8 clusters in the CyTOF. Data represents geometric MFI for each marker

1068   normalized to the minimum and maximum values across all CD8 clusters. Any markers

1069   exhibiting uniform high or low expression were normalized to the minimum and

1070   maximum values across all immune clusters to demonstrate high or low expression

1071   levels across clusters, respectively. **(D)** Violin plots representing relative proportions of

1072   CD8 clusters as a percentage of all CD8s in the indicated treatment groups. Two-way

1073   ANOVA with Tukey multiple testing correction was used to assess statistical

1074   significance. **(E)** Heatmap of relevant phenotypic marker expression across all tumor-

1075   infiltrating CD4 Tconv clusters in the CyTOF. Data represents geometric MFI for each

1076   marker normalized to the minimum and maximum values across all CD4 Tconv clusters.

1077   Any markers exhibiting uniform high or low expression were normalized to the minimum

1078   and maximum values across all immune clusters to demonstrate high or low expression

1079   levels across clusters, respectively. **(F)** Violin plots representing relative proportions of

1080   CD4 Tconv clusters as a percentage of all CD4 Tconv cells in the indicated treatment

1081   groups. Two-way ANOVA with Tukey multiple testing correction was used to assess

1082   statistical significance. **(G)** Heatmap of relevant phenotypic marker expression across

1083   all tumor-infiltrating CD8 clusters in the mouse spectral flow cytometry data. Data

1084   represents median MFI for each marker normalized to the minimum and maximum

1085   values across all CD8 clusters. Any markers exhibiting uniform high or low expression

1086   were normalized to the minimum and maximum values across all immune clusters to

1087   demonstrate high or low expression levels across clusters, respectively. **(D)** Violin plots

1088   representing relative proportions of CD8 clusters as a percentage of all CD8s in the

1089   indicated treatment groups. Two-way ANOVA with Tukey multiple testing correction was

1090   used to assess statistical significance. **(I)** Violin plots representing the frequency of

1091   CD39+41BB+ CD8s as a percentage of tumor-infiltrating CD8s (left) or as a percentage

1092   of CD45 (right) in a second independent replicate experiment. Student's t-test was used

1093   to assess statistical significance. **(J)** Violin plots representing the frequency of

1094   CD44$^+$Ki67$^+$ CD8s as a percentage of tdLN CD8s (left) or CD44$^+$Ki67$^+$ CD4 Tconv cells

1095   as a percentage of tdLN CD4 Tconv cells (right) in a second independent replicate

1096   experiment. Student's t-test was used to assess statistical significance. **(K)** Violin plots

1097   representing the frequency of Tregs as a percentage of all tdLN CD45$^+$ cells stratified by

1098   treatment group. Student's t-test was used to assess statistical significance.

49

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Figure 1

Figure 1

Figure 2

Figure 3

A

CyTOF
TI-Treg Phenotyping

Tr.1 - Memory-like
Tr.2 - Proliferating
Tr.3 - Cytotoxic
Tr.4 - Peripherally Induced
Tr.5 - CD39-
Tr.6 - CCR8-
Tr.7 - Resting
Tr.8 - HLA-DR+ Activated
Tr.9 - 4-1BB+ Activated

B — Helios, Ki67, GzmB, CD39, CCR8, CTLA-4, HLA-DR, 4-1BB; gMFI Max/Min

C — Normalized gMFI (0 0.2 0.4 0.6 0.8 1.0)

D — Untreated / ADT / ADT + anti-CTLA4-NF

E — **TI-Treg Composition**; % of Tregs; ADT: - + +; anti-CTLA4-NF: - - +

F — **CyTOF**: TI-Treg Relative Marker Expression — ADT + anti-CTLA4-NF vs ADT

G — **scRNAseq**: Tregs — ADT + anti-CTLA4-NF only; p=0.03

H — **scRNAseq Tregs**: Time-to-Recurrence — ADT + anti-CTLA4-NF only; p=0.0008; Treg-Low; Treg-High

Figure 4

Figure 5

Extended Data Figure 1

Extended Data Figure 2

Extended Data Figure 3

A

B

Extended Data Figure 4

Extended Data Figure 5

A — Mouse 45c Flow: **Tumor** Live CD45+ Gating

B — Mouse 45c Flow: **Tumor Draining Lymph Node** Live CD45+ Gating

C — Normalized Median MFI heatmap (DC.1–DC.6)

DC.1: Resting cDC1
DC.2: Activated cDC1
DC.3: cDC2
DC.4: iNOS+ DC3
DC.5: CD40+ DC3
DC.6: CD40- DC3

D — **Mouse Flow**: DC Cluster Frequencies

E — PD-L2+ DCs

Extended Data Figure 6

A scRNAseq: CD8 clusters (CD8-1 & CD8-2)

B *Cd8a* gene expression; *Cd4* gene expression; *CCR7* VIPER activity; Naive CD8 signature *Zheng et al. 2021*

C CyTOF: CD8 cluster phenotyping

Tc.1: Memory-like CCR8+
Tc.2: Resting/bystander
Tc.3: PD1+ effector
Tc.4: Effector memory
Tc.5: CD39+41BB+ effector
Tc.6: GzmB+Ki67+ effector
Tc.7: CD27- terminal effector
Tc.8: Terminally exhausted

D CyTOF: CD8 Clusters Frequencies

E CyTOF: CD4 Tconv cluster phenotyping

Tn.1: Memory-like
Tn.2: Naive CCR8+
Tn.3: Resting memory-like
Tn.4: CD39+41BB+ effector
Tn.5: CTLA4+PD1+ effector
Tn.6: CD27- memory-like
Tn.7: GzmB+ effector
Tn.8: Ki67+ effector

F CyTOF: CD4 Tconv Cluster Frequencies

G Mouse Flow: CD8 cluster phenotyping

Tc.1: CD62L+ naive
Tc.2: Ly6C- EM
Tc.3: Ly6C+ naive
Tc.4: Ly6C+ EM
Tc.5: PD1- early effector
Tc.6: PD1+Ki67- effector
Tc.7: PD1+Ki67+ effector
Tc.8: CD69+CD103+ Trm
Tc.9: CD39+41BB+ effector
Tc.10: Terminally exhausted

H Mouse Flow: CD8 Cluster Frequencies

I Tumor CD8

J tdLN CD8 / tdLN CD4 Tconv

K tdLN Tregs