

1 **$\gamma\delta$ T cells are effectors of immune checkpoint blockade in mismatch repair-deficient**
2 **colon cancers with antigen presentation defects**

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22 **Running title:** $\gamma\delta$ T cells mediate responses to ICB in MMR-deficient cancers

23

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26 **Abstract**

27 DNA mismatch repair deficient (MMR-d) cancers present an abundance of neoantigens that
28 likely underlies their exceptional responsiveness to immune checkpoint blockade (ICB)^{1,2}.
29 However, MMR-d colon cancers that evade CD8⁺ T cells through loss of Human Leukocyte
30 Antigen (HLA) class I-mediated antigen presentation³⁻⁶, frequently remain responsive to ICB⁷
31 suggesting the involvement of other immune effector cells. Here, we demonstrate that HLA
32 class I-negative MMR-d cancers are highly infiltrated by $\gamma\delta$ T cells. These $\gamma\delta$ T cells are mainly
33 composed of V δ 1 and V δ 3 subsets, and express high levels of PD-1, activation markers
34 including cytotoxic molecules, and a broad repertoire of killer-cell immunoglobulin-like
35 receptors (KIRs). *In vitro*, PD-1⁺ $\gamma\delta$ T cells, isolated from MMR-d colon cancers, exhibited a
36 cytolytic response towards HLA class I-negative MMR-d colon cancer cell lines and β 2-
37 *microglobulin* (*B2M*)-knockout patient-derived tumor organoids (PDTOs), which was
38 enhanced as compared to antigen presentation-proficient cells. This response was diminished
39 after blocking the interaction between NKG2D and its ligands. By comparing paired tumor
40 samples of MMR-d colorectal cancer patients obtained before and after dual PD-1 and CTLA-
41 4 blockade, we found that ICB profoundly increased the intratumoral frequency of $\gamma\delta$ T cells in
42 HLA class I-negative cancers. Taken together, these data indicate that $\gamma\delta$ T cells contribute to
43 the response to ICB therapy in patients with HLA class I-negative, MMR-d colon cancers, and
44 illustrate the potential of $\gamma\delta$ T cells in cancer immunotherapy.

45 Introduction

46 Immune-checkpoint blockade (ICB) targeting the PD-1/PD-L1 and/or CTLA-4 axis provides
47 durable clinical benefit to patients with DNA mismatch repair-deficient (MMR-d)/Microsatellite
48 Instability-High (MSI-H) cancers⁸⁻¹¹. The exceptional responses of MMR-d/MSI-H cancers to
49 ICB are likely explained by their vast burden of putative neoantigens, which originate from the
50 extensive accumulation of mutations in their genomes^{1,2}. This is in line with the current view
51 that PD-1 blockade mainly boosts endogenous antitumor immunity driven by CD8⁺ T cells,
52 which recognize Human Leukocyte Antigen (HLA) class I-bound neoepitopes on cancer
53 cells¹²⁻¹⁴. However, MMR-d colon cancers frequently lose HLA class I-mediated antigen
54 presentation due to silencing of HLA class I genes, inactivating mutations in *β2-microglobulin*
55 (B2M), or other defects in the antigen processing machinery³⁻⁶, which may render these
56 tumors resistant to CD8⁺ T cell-mediated immunity. Interestingly, the majority of β2m-deficient
57 MMR-d cancers have shown durable responses to PD-1 blockade⁷, suggesting that immune
58 cell subsets other than CD8⁺ T cells contribute to these responses.

59 Immune cell subsets capable of HLA class I-independent tumor killing include natural
60 killer (NK) cells and $\gamma\delta$ T cells. $\gamma\delta$ T cells share many characteristics with their $\alpha\beta$ T cell
61 counterpart, such as cytotoxic effector functions, but express a distinct TCR composed of a γ
62 and δ chain. Different subsets of $\gamma\delta$ T cells are defined by their TCR δ chain usage, of which
63 those expressing V δ 1 and V δ 3 are primarily “tissue-resident” at mucosal sites, whereas those
64 expressing V δ 2 are mainly found in blood¹⁵. Both adaptive and innate mechanisms of
65 activation, e.g., through stimulation of their $\gamma\delta$ TCR or innate receptors such as NKG2D,
66 DNAM-1, NKp30 or NKp44, have been described for $\gamma\delta$ T cells¹⁶. Killer-cell immunoglobulin-
67 like receptors (KIRs) are expressed by $\gamma\delta$ T cells and regulate their activity depending on HLA
68 class I expression¹⁷. Furthermore, $\gamma\delta$ T cells were found to express high levels of PD-1 in
69 MMR-d colorectal cancers (CRCs), suggesting that these cells may be targeted by PD-1
70 blockade¹⁸.

71 Here, we applied a combination of transcriptomic and imaging approaches for an in-
72 depth analysis of ICB-naïve and ICB-treated MMR-d colon cancers, as well as *in vitro*
73 functional assays, and found evidence indicating that $\gamma\delta$ T cells mediate responses to HLA
74 class I-negative, MMR-d tumors during ICB therapy.

75

76 Results

77 $\gamma\delta$ T cells are enriched in *B2M*-mutant MMR-d cancers

78 To gain insights into immune cell subsets involved in immune responses towards HLA class
79 I-negative MMR-d cancers, we studied the transcriptomic changes associated with genomic
80 loss of *B2M* in three MMR-d cancer cohorts in The Cancer Genome Atlas (TCGA): colon
81 adenocarcinoma (COAD; n=44 *B2M*^{WT}, n=6 *B2M*^{MUT}), stomach adenocarcinoma (STAD; n=48
82 *B2M*^{WT}, n=13 *B2M*^{MUT}), and endometrium carcinoma (UCEC; n=99 *B2M*^{WT}, n=3 *B2M*^{MUT}). We
83 found that *B2M* was among the most significantly downregulated genes in *B2M*^{MUT} tumors
84 (two-sided P=8.4x10⁻⁵, Benjamini-Hochberg corrected false discovery rate [FDR]=0.040; [Fig.](#)
85 [1a](#)). Genes encoding components of the HLA class I antigen presentation machinery other
86 than $\beta 2m$ were highly upregulated in *B2M*^{MUT} tumors ([Fig. 1a](#)). Interestingly, we found *TRDV1*
87 and *TRDV3*, which encode the variable regions of the $\delta 1$ and $\delta 3$ chains of the $\gamma\delta$ T cell receptor
88 (TCR), among the most significantly upregulated loci in *B2M*^{MUT} tumors (*TRDV1*: FDR=0.0056;
89 *TRDV3*: FDR=0.0062; [Fig. 1a](#)). In line with this, the expression level of $\gamma\delta$ TCRs was
90 significantly higher in *B2M*^{MUT} compared to *B2M*^{WT} MMR-d cancers (Wilcoxon rank sum-based
91 two-sided P=1.1x10⁻⁵ for all cohorts combined; [Fig. 1b](#)), while this was less pronounced for $\alpha\beta$
92 TCR expression (Wilcoxon rank sum-based two-sided P=0.023 for all cohorts combined;
93 [Extended Data Fig. 1](#)). In addition, multiple KIRs showed clear overexpression in *B2M*^{MUT}
94 tumors ([Fig. 1a](#)), where the expression level of all human KIRs combined was significantly
95 higher in *B2M*^{MUT} compared to *B2M*^{WT} MMR-d tumors (Wilcoxon rank sum-based two-sided
96 P=3.0x10⁻⁷ for all cohorts combined; [Fig. 1c](#)). Together, these results suggest that ICB-naïve,
97 *B2M*^{MUT} MMR-d cancers show increased levels of (1) V $\delta 1$ and V $\delta 3$ T cells and (2) immune
98 cells expressing KIRs, receptors implied in the recognition and killing of HLA class I-negative
99 cells.

100 We next assessed if the upregulation of $\gamma\delta$ TCRs and KIRs in *B2M*^{MUT} MMR-d tumors
101 was caused by higher overall levels of cellular infiltration. To this end, we used marker gene
102 sets¹⁹ to estimate the abundance of a broad set of immune cell types based on the RNA
103 expression data of the TCGA cohorts. Hierarchical clustering identified a highly and a lowly
104 infiltrated cluster in each of the three tumor types ([Fig. 1d](#)). Apart from the $\gamma\delta$ TCR and KIR
105 gene sets, none of the other marker gene sets showed increased expression in *B2M*^{MUT} versus
106 *B2M*^{WT} tumors ([Fig. 1d](#)).

107 Imaging mass cytometry analysis of MMR-d colon cancers, with HLA class I-loss due
108 to defects in $\beta 2m$, revealed that $\gamma\delta$ T cells frequently displayed an intraepithelial localization
109 and expression of CD103 (tissue-residency), CD39 (activation), granzyme B (cytotoxicity), and

110 Ki-67 (proliferation), as well as PD-1 (Fig. 1e). Interestingly, $\beta 2m^-$ cancers showed a
111 significantly increased fraction of CD103⁺CD39⁺ $\gamma\delta$ T cells as compared to HLA class I⁺
112 cancers (two-sided P=0.0307 by Kruskal-Wallis test; Fig. 1f). Co-expression of CD103 and
113 CD39 was reported to identify tumor-reactive CD8⁺ $\alpha\beta$ T cells in a variety of cancers²⁰.
114 Altogether, these data support a role for $\gamma\delta$ T cells in mediating natural cytotoxic antitumor
115 responses in HLA class I-negative MMR-d colon cancers.

116 **Cytotoxic V δ 1 and V δ 3 $\gamma\delta$ T cells infiltrate MMR-d colon cancers**

117 To investigate which $\gamma\delta$ T cell subsets are present in MMR-d colon cancers and to determine
118 their functional characteristics, we performed single-cell RNA-sequencing on $\gamma\delta$ T cells
119 isolated from five MMR-d colon cancers (Extended Data Fig. 2, Extended Data Table 1). Three
120 distinct V δ subsets were identified (Fig. 2a, Extended Data Fig. 3), where V δ 1 T cells were the
121 most prevalent (43% of $\gamma\delta$ T cells), followed by V δ 2 (19%) and V δ 3 T cells (11%) (Fig. 2b).
122 *PDCD1* (encoding PD-1) was predominantly expressed by V δ 1 and V δ 3 $\gamma\delta$ T cells, while V δ 1
123 cells expressed high levels of genes encoding activation markers such as CD39 (*ENTPD1*)
124 and CD38 (Fig. 2c, Extended Data Fig. 2). Furthermore, proliferating $\gamma\delta$ T cells (expressing
125 *MKI67*) were especially observed in the V δ 1 and V δ 3 subsets (Fig. 2c). Other distinguishing
126 features of V δ 1 and V δ 3 T cell subsets included the expression of genes encoding activating
127 receptors NKp46 (*NCR1*), NKG2C (*KLRC2*), and NKG2D (*KLRK1*) (Fig. 2c). Interestingly, the
128 expression of several KIRs was also higher in the V δ 1 and V δ 3 subsets as compared to V δ 2
129 T cells (Fig. 2c). Almost all $\gamma\delta$ T cells displayed expression of genes encoding Granzyme B
130 (*GZMB*), Perforin (*PRF1*), and Granulysin (*GNL1*) (Fig. 2c).

131 **PD-1⁺ $\gamma\delta$ T cells are cytotoxic towards HLA class I-negative colon cancer cells**

132 We next sought to determine whether tumor-infiltrating $\gamma\delta$ T cells can recognize and kill CRC
133 cells. We isolated and expanded PD-1⁻ and PD-1⁺ $\gamma\delta$ T cells (Extended Data Fig. 4) from five
134 MMR-d colon cancers (Extended Data Table 1). In line with the scRNA-seq data, expanded
135 PD-1⁺ $\gamma\delta$ T cells were devoid of V δ 2⁺ cells and comprised of V δ 1⁺ or V δ 3⁺ subsets, whereas
136 PD-1⁻ fractions contained V δ 2⁺ or a mixture of V δ 1/V δ 2/V δ 3⁺ populations (Fig. 3a, Extended
137 Data Fig. 4). Detailed immunophenotyping of the expanded $\gamma\delta$ T cells showed that all cells
138 expressed the activating receptor NKG2D, while the surface expression of KIRs was most
139 frequent on PD-1⁺ $\gamma\delta$ T cells (V δ 1 or V δ 3⁺), in line with the scRNA-seq results of unexpanded
140 populations (Fig. 3a, Extended Data Fig. 5).

141 We measured the reactivity of the expanded $\gamma\delta$ T cell populations towards HLA class
142 I-negative and HLA class I-positive cancer cell lines (Fig. 3b, Extended Data Fig. 5). Upon co-
143 culture with the different cancer cell lines, expression of activation markers and secretion of
144 IFN γ was mainly induced in PD-1⁺ $\gamma\delta$ T cells (V δ 1 or V δ 3⁺) and cell reactivity was most
145 pronounced against HLA class I-negative cell lines (Fig. 3c, Extended Data Fig. 5-6).
146 Reactivity of PD-1⁻ (enriched in V δ 2⁺) subsets towards colorectal cancer cell lines was not
147 detected (Fig. 3c, Extended Data Fig. 5-6). To quantify and visualize the differences in killing
148 of CRC cell lines by PD-1⁺ and PD-1⁻ $\gamma\delta$ T cells, we co-cultured the $\gamma\delta$ T cell populations with
149 three CRC cell lines (HCT-15, LoVo, HT-29) transduced with a fluorescent caspase-3/7
150 reagent to measure cancer cell apoptosis over time (Fig. 3d-e). This showed pronounced
151 cancer cell apoptosis upon co-culture with PD-1⁺ $\gamma\delta$ T cells (V δ 1 or V δ 3⁺) compared to PD-1⁻
152 cells, with highest killing of HLA class I-negative HCT-15 cells (Fig. 3e, Movie 1-2).

153 Next, we established two parental patient-derived tumor organoid lines (PDTOs;
154 Extended Data Table 2) of MMR-d CRC and generated isogenic *B2M*^{KO} lines using CRISPR.
155 Genomic knockout of *B2M* effectively abrogated cell surface expression of HLA class
156 I (Extended Data Fig. 7). We exposed two *B2M*^{KO} and their parental *B2M*^{WT} lines to the
157 expanded $\gamma\delta$ T cell subsets, and quantified $\gamma\delta$ T cell activation by determination of IFN γ
158 expression. Similarly to our cell line data, $\gamma\delta$ T cells displayed increased reactivity towards
159 *B2M*^{KO} PDTOs in comparison to the *B2M*^{WT} PDTOs (Fig. 3f-g). Furthermore, $\gamma\delta$ T cell reactivity
160 towards *B2M*^{KO} tumor organoids was preferentially contained within the PD-1⁺ population of
161 $\gamma\delta$ T cells (Fig. 3g). Thus, lack of HLA class I antigen presentation in MMR-d tumor cells can
162 be effectively sensed by $\gamma\delta$ T cells and stimulates their antitumor response.

163 Expression of NKG2D on $\gamma\delta$ T cells decreased during co-culture with target cells
164 (Extended Data Fig. 8), indicating the involvement of the NKG2D receptor in $\gamma\delta$ T cell activity.
165 The NKG2D ligands MICA/B and ULBPs were expressed by the cancer cell lines (Fig. 3b) and
166 the MMR-d CRC PDTOs, irrespective of their *B2M* status (Extended Data Fig. 7). To explore
167 which receptor-ligand interactions might regulate the activity of PD-1⁺ $\gamma\delta$ T cells, we performed
168 blocking experiments focused on (i) NKG2D, (ii) DNAM-1, and (iii) $\gamma\delta$ TCR signaling. Of these
169 candidates, the only consistent inhibitory effect was observed for NKG2D ligand blocking on
170 cancer cells, which decreased the activation and killing capacity of most PD-1⁺ $\gamma\delta$ T cells (Fig.
171 3h, Extended Data Fig. 9-10), confirming the mechanistic involvement of the NKG2D receptor
172 in $\gamma\delta$ T cell activation in this context. In addition, blocking NKG2D ligands on MMR-d CRC
173 PDTOs reduced the PDTO-directed tumor reactivity of $\gamma\delta$ T cells from CRC94 and CRC134

174 (Fig. 3i). Together, these results show that $\gamma\delta$ T cell reactivity towards MMR-d tumors is partly
175 dependent on NKG2D/NKG2D-ligand interactions.

176 **Activated $\gamma\delta$ T cells infiltrate ICB-treated *B2M*-mutant MMR-d colon cancers**

177 Next, we studied how ICB influences $\gamma\delta$ T cell infiltration and activation in MMR-d colon
178 cancers in a therapeutic context. For this purpose, we analysed pre- and post-treatment
179 samples of the NICHE trial¹¹, in which colon cancer patients were treated with neoadjuvant
180 PD-1 plus CTLA-4 blockade. In MMR-d colon cancers (n=38), genes encoding $\gamma\delta$ TCRs were
181 highly upregulated in response to ICB (Fig. 4a). When specifically focusing on the five *B2M*^{MUT}
182 cancers, we found that expression of genes encoding $\gamma\delta$ TCRs was also strongly induced upon
183 ICB, with consistently larger effect sizes as compared to *B2M*^{WT} cancers (Fig. 4b). Of the δ
184 variable regions, expression of *TRDV1* (V δ 1) was most strongly induced upon ICB in *B2M*^{MUT}
185 cancers (Fig. 4c). The expression of KIRs was also upregulated upon treatment with ICB, both
186 in the cohort as a whole, and in the subgroup of *B2M*^{MUT} cancers (Fig. 4a-b). The set of KIRs
187 upregulated upon ICB in *B2M*^{MUT} cancers (Fig. 4d) was consistent with the sets of KIRs
188 upregulated in *B2M*^{MUT} MMR-d cancers in TCGA (Fig. 1a), and those expressed by MMR-d
189 tumor-infiltrating $\gamma\delta$ T cells (Fig. 2c). Finally, expression of the $\gamma\delta$ TCR and KIR gene sets was
190 more strongly induced upon response to ICB in *B2M*^{MUT} versus *B2M*^{WT} cancers ($\gamma\delta$ TCRs: two-
191 sided interaction P=0.0056; KIRs: two-sided interaction P=0.047 Fig. 4e).

192 To quantify and investigate differences in phenotype of $\gamma\delta$ T cells upon ICB treatment,
193 we applied imaging mass cytometry to profile immune cell infiltration in post-ICB tissues
194 derived from five *B2M*^{MUT} HLA class I-negative and five *B2M*^{WT} HLA class I-positive cancers.
195 Due to major pathologic clinical responses, residual cancer cells were absent in most post-
196 ICB treatment samples. All tissues showed a profound infiltration of different types of immune
197 cells, of which $\gamma\delta$ T cell infiltration was significantly increased in ICB-treated *B2M*^{MUT} as
198 compared to *B2M*^{WT} MMR-d colon cancers (two-sided P=0.0079 by Mann-Whitney test; Fig.
199 4f, Extended Data Fig. 11). In the sole *B2M*^{MUT} case that contained cancer cells, $\gamma\delta$ T cells
200 displayed co-expression of CD103, Ki-67, CD39, granzyme B, and PD-1 (Fig. 4g).
201 Furthermore, we observed that $\gamma\delta$ T cells directly interacted with caspase-3⁺ apoptotic cancer
202 cells in this tumor (Fig. 4h). Taken together, these results show that ICB treatment of MMR-d
203 colon cancer profoundly increases the intratumoral presence of activated, cytotoxic and
204 proliferating $\gamma\delta$ T cells, especially when these cancers are β 2m-deficient, pointing out $\gamma\delta$ T cells
205 as effectors of ICB treatment within this context.

206 Discussion

207 CD8⁺ $\alpha\beta$ T cells are major effectors of ICB^{13,14,21}, but the clinically relevant responses observed
208 in MMR-d cancers with HLA class I- defects suggest the involvement of other immune effector
209 cells. Here, we show that genomic inactivation of *B2M* in MMR-d colon cancers was
210 associated with: (i) an elevated frequency of activated $\gamma\delta$ T cells in ICB-naïve tumors, (ii) an
211 increased presence of tumor-infiltrating $\gamma\delta$ T cells upon ICB treatment, (iii) *in vitro* activation of
212 tumor-infiltrating $\gamma\delta$ T cells by colorectal cancer cell lines and PDO, and iv) killing of these
213 tumor cells by $\gamma\delta$ T cells, in particular by V δ 1 and V δ 3 subsets expressing PD-1.

214 Different subsets of $\gamma\delta$ T cells exhibit remarkably diverse functions which, in the context
215 of cancer, ranges from tumor-promoting to tumoricidal effects.^{22,23} Hence, it is of interest what
216 defines antitumor reactivity of $\gamma\delta$ T cells. Our data suggest that especially tumor-infiltrating V δ 1
217 and V δ 3 T cells can recognize and kill HLA class I-negative MMR-d tumors, whereas V γ 9V δ 2
218 cells, the most studied and main subset of $\gamma\delta$ T cells in the blood, appear to be less relevant
219 within this context. This is in line with other studies showing that the cytotoxic ability of V δ 1
220 cells generally outperforms their V δ 2 counterparts²⁴⁻²⁸. Of note, cytotoxicity of tumor-infiltrating
221 V δ 3 cells has, to our knowledge, not been reported before. Furthermore, the observation that
222 PD-1⁺ $\gamma\delta$ T cells demonstrated clearly higher levels of antitumor reactivity as compared to their
223 PD-1⁻ counterparts suggests that, as for CD8⁺ $\alpha\beta$ T cells²⁹, PD-1 expression may be a marker
224 of antitumor reactivity in $\gamma\delta$ T cells.

225 The mechanisms of activation of $\gamma\delta$ T cells are notoriously complex and diverse¹⁶.
226 Specifically for V δ 1⁺ cells, NKG2D has been described to be involved in tumor recognition,
227 which is dependent on tumor cell expression of NKG2D ligands MICA/B and ULBPs^{30,31}. In
228 our study, MICA/B and ULBPs were highly expressed by the MMR-d CRC cell lines and tumor
229 organoids, and blocking these ligands reduced $\gamma\delta$ T cell activation and cytotoxicity. This
230 suggests a role for the activation receptor NKG2D in $\gamma\delta$ T cell reactivity towards HLA class I-
231 negative MMR-d tumors. In addition, we detected expression of KIRs primarily on PD-1⁺ $\gamma\delta$ T
232 cells (V δ 1 or V δ 3⁺ subsets), whose antitumor reactivity and killing was clearly amplified when
233 tumor cells lacked HLA class I.

234 Our findings have broad implications for cancer immunotherapy. First, our results
235 suggest that MMR-d cancers and other tumors with HLA class I defects may be particularly
236 attractive targets for V δ 1 or V δ 3 $\gamma\delta$ T cell-based cellular therapies. Second, our findings provide
237 a basis for novel (combinatorial) immunotherapeutic approaches to further enhance $\gamma\delta$ T cell-
238 based antitumor immunity. Third, the presence or absence in tumors of specific $\gamma\delta$ T cell

239 subsets (e.g. V δ 1 or V δ 3) may help to define patients (un)responsive to ICB, especially in the
240 case of MMR-d cancers and other malignancies with frequent HLA class I defects, like
241 stomach adenocarcinoma³² and Hodgkin lymphoma³³.

242 Although we have provided detailed and multidimensional analyses, our study is
243 relatively small and it is conceivable that $\gamma\delta$ T cells are not the only factor driving ICB responses
244 in HLA class I-negative MMR-d CRC tumors. In this context, other HLA class I-independent
245 immune subsets, like innate lymphoid cells (ILCs), (neoantigen-specific) CD4⁺ T cells (as
246 reported in murine MMR-d cancer models³⁴), and macrophages may also contribute. In
247 addition, the requirements of other immune cell types in providing help for effector functions
248 of $\gamma\delta$ T cells needs to be clarified.

249 In conclusion, our results provide strong evidence that $\gamma\delta$ T cells are cytotoxic effector
250 cells of ICB treatment in HLA class I-negative MMR-d colon cancers, with implications for
251 further exploitation of $\gamma\delta$ T cells in cancer immunotherapy.

252 Main references

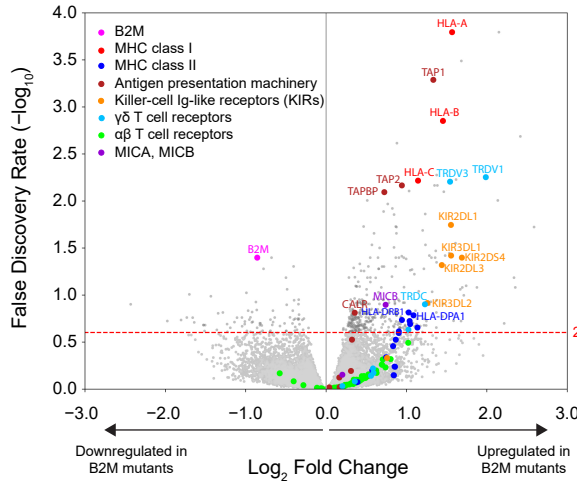
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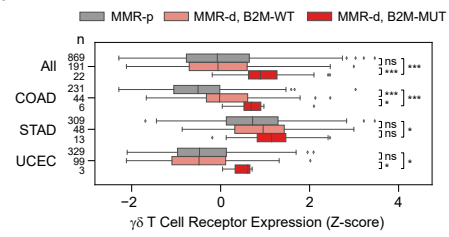
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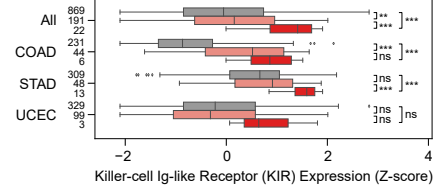
Figure 1
a



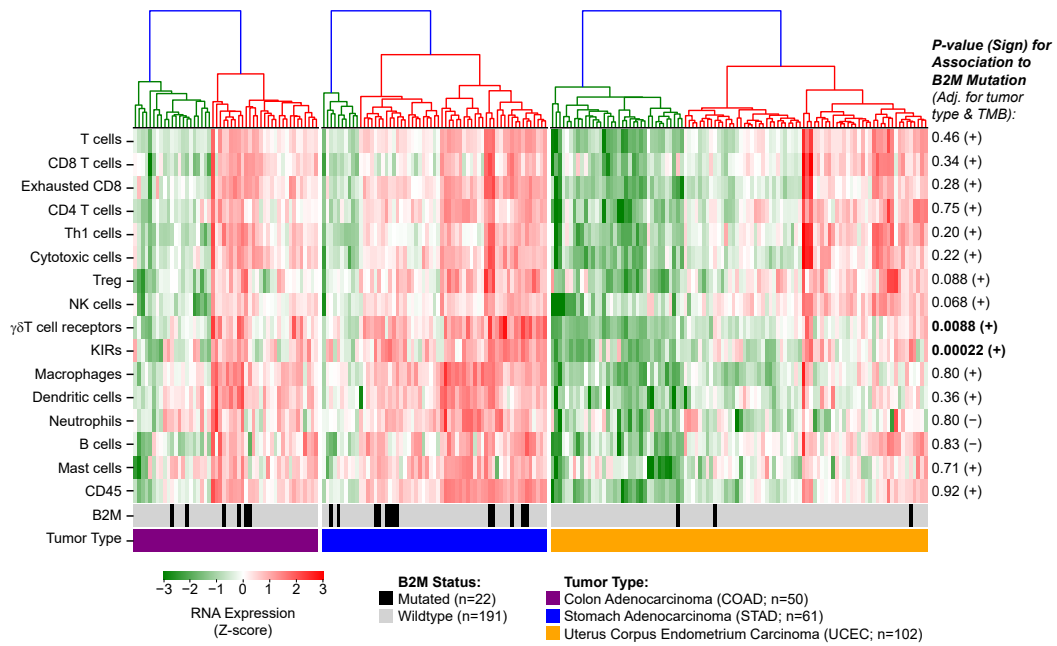
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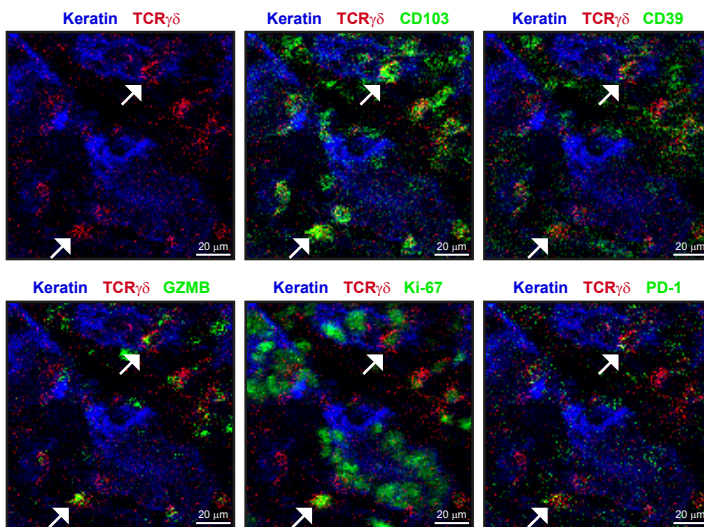
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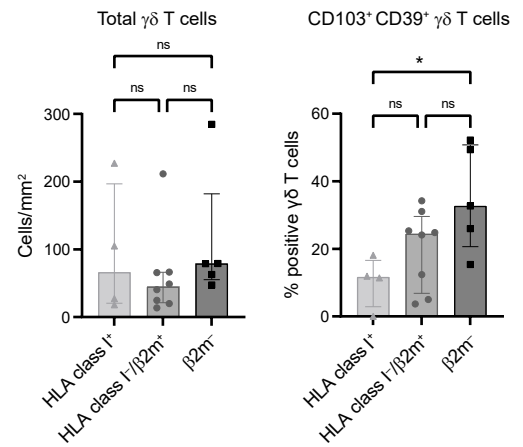


Figure 1. $\beta 2m$ defects are associated with increased infiltration of MMR-d cancers by $\gamma\delta$ T cells and killer-cell immunoglobulin-like receptor (KIR)-expressing cells.

a. Volcano plot indicating differential gene expression between MMR-d cancers with vs. without high impact inactivating mutations in *B2M*. The Benjamini Hochberg false discovery rate (FDR) significance threshold of 25% is indicated by the red dashed line. Results were obtained in a combined analysis on the TCGA COAD, STAD and UCEC cohorts, and were adjusted for tumor type and tumor mutational burden.

b. Boxplot showing the RNA expression of $\gamma\delta$ T cell receptors in MMR-p (gray), MMR-d *B2M*^{WT} (pink), and MMR-d *B2M*^{MUT} (red) cancers. Results are obtained with the TCGA COAD, STAD and UCEC cohorts, and are shown for all cohorts combined (All), and for each cohort separately. Boxes, whiskers, and dots indicate quartiles, 1.5 interquartile ranges, and outliers, respectively. P-values were calculated by Wilcoxon rank sum test. * P<0.05; ** P<0.01; *** P<0.001.

c. As (b), but for RNA expression of killer-cell immunoglobulin-like receptors (KIRs).

d. Heatmap of the expression (Z-score; see color bar) of gene sets whose expression marks infiltration of specific immune cell types in MMR-d cancers of the COAD, STAD and UCEC cohorts of TCGA. Cancers were ranked based on hierarchical clustering, as indicated by the dendrograms (top). The lower two bars indicate the *B2M* mutation status and cancer type, as defined in the legend. P-values and sign (+ for positive and – for negative) of associations of the expression of each marker gene set with *B2M* mutation status are shown on the right. P-values were obtained by ordinary least squares linear regression and adjusted for tumor type and tumor mutational burden. Significant associations are in bold font.

e. Representative images of the detection of tissue-resident (CD103⁺), activated (CD39⁺), cytotoxic (granzyme B⁺), proliferating (Ki-67⁺), and PD-1⁺ $\gamma\delta$ T cells by imaging mass cytometry in an ICB-naïve, MMR-d colon cancer with $\beta 2m$ defect.

f. Frequencies of total $\gamma\delta$ T cells and CD103⁺ CD39⁺ $\gamma\delta$ T cells in ICB-naïve HLA class I-positive (+) (n=4), HLA class I-negative (-)/ $\beta 2m$ ⁺ (n=8), and $\beta 2m$ ⁻ MMR-d colon cancers (n=5). Bars indicate median \pm IQR. Each dot represents an individual sample. P-values were calculated by Kruskal-Wallis test with Dunn's test for multiple comparisons. *P<0.05.

Figure 2

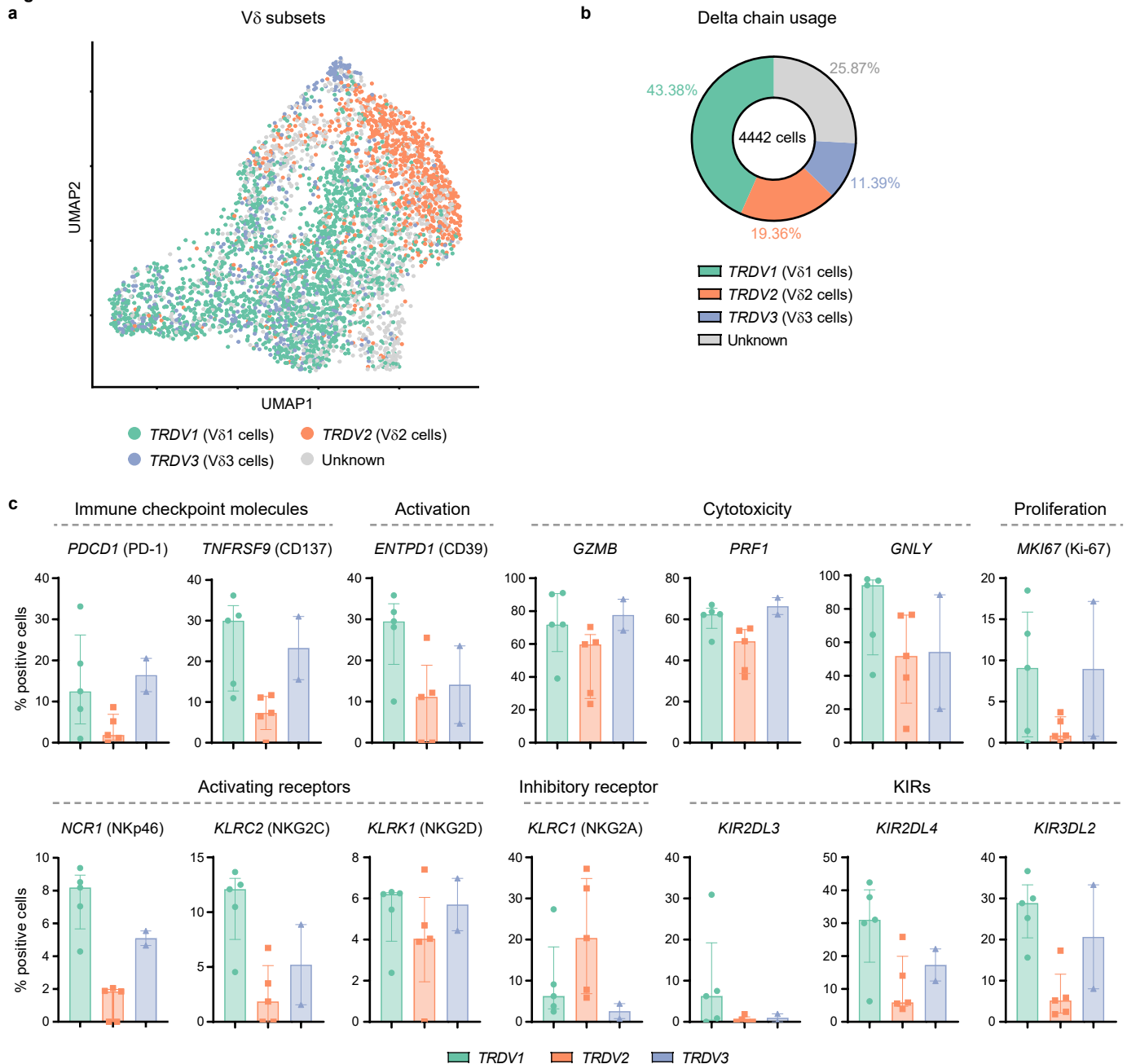


Figure 3

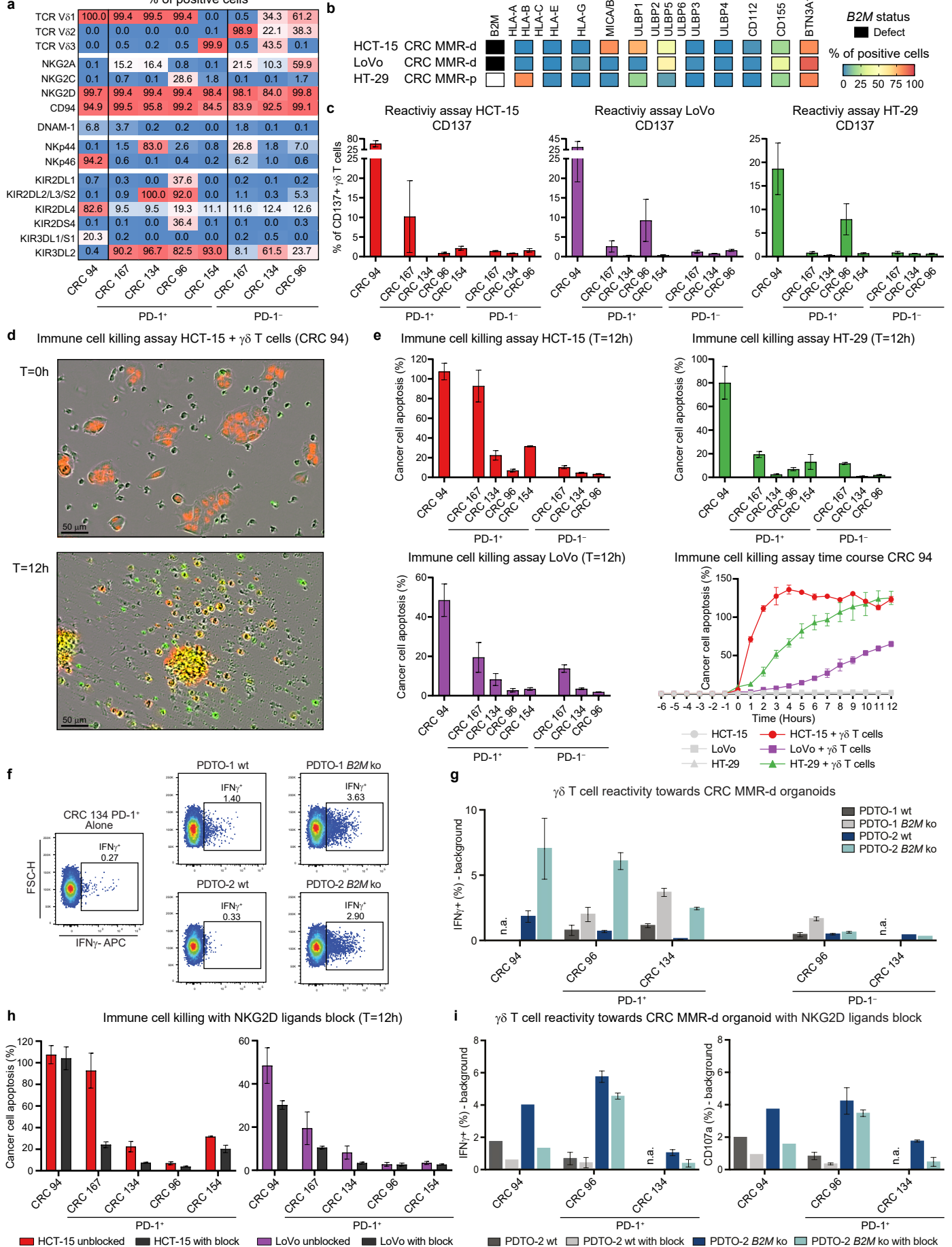


Figure 3. $\gamma\delta$ T cells from MMR-d colon cancers show preferential reactivity towards HLA class I-negative cancer cell lines and organoids, which is regulated by NKG2D/NKG2D ligand interactions.

a. Table showing the percentage of positive cells for different TCR V δ chains, innate immune receptors, and KIRs on expanded PD-1⁺ and PD-1⁻ $\gamma\delta$ T cells sorted from MMR-d colon cancers (n=5) as percentage of total $\gamma\delta$ T cells.

b. Diagram showing the *B2M* mutational status and surface expression of HLA class I, NKG2D ligands, DNAM-1 ligands, and butyrophilin on CRC cell lines HCT-15, LoVo, and HT-29.

c. Bar plots showing the percentage of CD137-positive $\gamma\delta$ T cells after 18h co-culture of PD-1⁺ and PD-1⁻ $\gamma\delta$ T cells from MMR-d colon cancers (n=5) with HCT-15, LoVo, and HT-29 cells. Medium as negative control and PMA/ionomycin as positive control are shown in Extended Data Fig. 5. Bars indicate mean \pm SEM. Data from four (CRC94), three (CRC167, CRC96), or two (CRC134, CRC154) independent experiments, depending on availability of $\gamma\delta$ T cells.

d. Representative images showing the killing of NuCLight Red-transduced HCT-15 cells by $\gamma\delta$ T cells (unlabeled) from CRC94 in the presence of a green fluorescent caspase-3/7 reagent in the IncuCyte S3. Images are taken immediately after the addition of $\gamma\delta$ T cells (T=0) and 12h after. Cancer cell apoptosis is visualized in yellow.

e. Bar plots showing the quantification of the killing of cancer cell lines by $\gamma\delta$ T cells from MMR-d colon cancers (n=5) as in (d) after 12h co-culture. Bars indicate mean \pm SEM of two wells with two images/well. At lower right, representative time course of cancer cell apoptosis in the presence or absence of $\gamma\delta$ T cells derived from CRC94.

f. Representative flow cytometry plots of PD-1⁺ $\gamma\delta$ T cells from CRC134 indicating IFN γ expression in unstimulated condition (alone) and upon stimulation with two *B2M*^{WT} and *B2M*^{KO} CRC MMR-d organoids, as specified in the subplot titles.

g. Histogram showing IFN γ expression of $\gamma\delta$ T cells from MMR-d colon cancers upon stimulation with two *B2M*^{WT} and *B2M*^{KO} CRC MMR-d organoids, as specified in the legend. Background IFN γ signal of each unstimulated $\gamma\delta$ T cell sample was subtracted from tumor organoid-stimulated IFN γ signal. For all $\gamma\delta$ T cell samples, data is shown for two biological replicates except for CRC134 PD-1⁻ (n=1). Whiskers indicate SEM.

h. Bar plots showing the quantification of the killing of cancer cell lines by $\gamma\delta$ T cells from MMR-d colon cancers (n=5) in the presence of blocking antibodies for NKG2D ligands as compared to the unblocked condition after 12h co-culture. Bars indicate mean \pm SEM of two wells with two images/well.

i. Histograms showing IFN γ (left) and CD107a (right) expression in $\gamma\delta$ T cells from MMR-d colon cancers upon stimulation with *B2M*^{WT} PDO-2 (gray shades) or *B2M*^{KO} PDO-2 (blue shades), with or without NKG2D ligand blocking (as indicated in the legend). For cultured $\gamma\delta$ T cells, data is shown for two biological replicates (n=2) except for CRC94 (n=1). Whiskers indicate SEM.

Figure 4

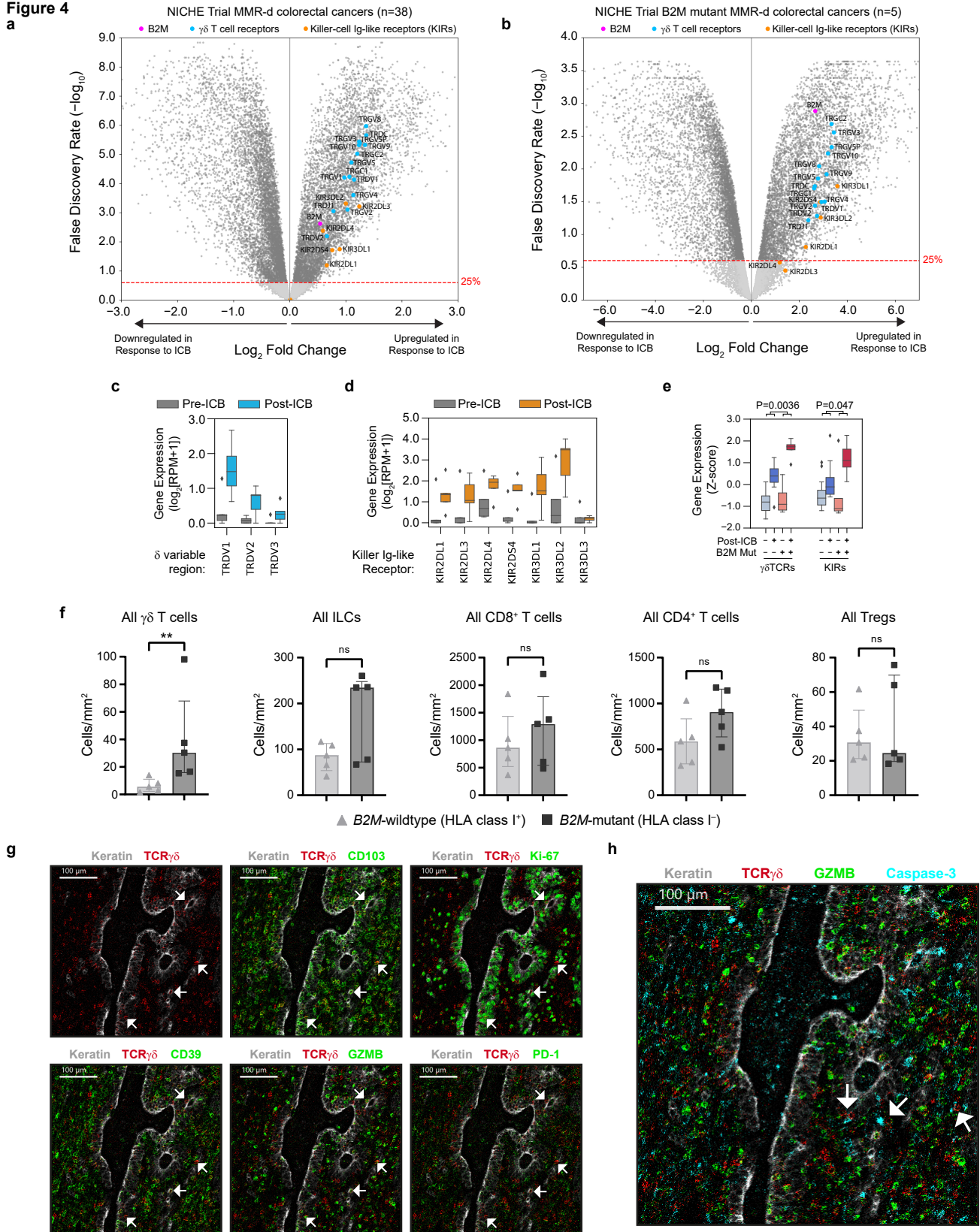


Figure 4. Immune checkpoint blockade (ICB) induces profound infiltration of $\gamma\delta$ T cells into MMR-d colon cancers with antigen presentation defects.

- a.** Volcano plot indicating differential RNA expression of genes between MMR-d cancers before and after ICB in the NICHE study. The Benjamini Hochberg FDR significance threshold of 25% is indicated by the red dashed line.
- b.** As (a), but restricted to the five MMR-d cancers in the NICHE study with high impact (inactivating) mutations in *B2M*.
- c.** Boxplot showing the pre- (gray) and post-ICB (blue) RNA expression of δ T cell receptor variable regions in MMR-d cancers in the NICHE study with high impact (inactivating) mutations in *B2M*. Boxes, whiskers, and dots indicate quartiles, 1.5 interquartile ranges, and outliers, respectively. P-values were calculated by Wilcoxon rank sum test.
- d.** As (c), but for killer-cell Ig-like receptors (KIRs; post-ICB in orange).
- e.** Boxplot showing the pre- and post-ICB RNA expression of $\gamma\delta$ T cell receptors and killer-cell Ig-like receptors for MMR-d cancers with and without high impact *B2M* mutations (as indicated in below the x-axis). P-values were for *B2M*_status:treatment interaction in an ordinary least squares linear regression model. Significant p-values indicate that the treatment-induced increase in $\gamma\delta$ TCR/KIR expression is more pronounced in *B2M*^{MUT} versus *B2M*^{WT} cancers. Boxes, whiskers, and dots indicate quartiles, 1.5 interquartile ranges, and outliers, respectively.
- f.** Frequencies of different immune cell populations in *B2M*^{WT} (HLA class I-positive, n=5) and *B2M*^{MUT} (HLA class I-negative, n=5) MMR-d colon cancers upon ICB treatment. Bars indicate median \pm IQR. Each dot represents an individual sample. P-values were calculated by Mann-Whitney test. **P<0.01.
- g.** Representative images of the detection of tissue-resident (CD103⁺), proliferating (Ki-67⁺), activated (CD39⁺), cytotoxic (GZMB⁺), and PD1⁺ $\gamma\delta$ T cells by imaging mass cytometry in a *B2M*^{MUT} MMR-d colon cancer upon ICB treatment.
- h.** Representative image showing the interaction between $\gamma\delta$ T cells (colored in red) and caspase-3⁺ cancer cells (colored in cyan) by imaging mass cytometry in a *B2M*^{MUT} MMR-d colon tumor upon ICB treatment.

354 **Methods**

355 **TCGA data**

356 RNA expression data (raw counts and Fragments Per Kilobase of transcript per Million
357 mapped reads upper quartile FPKM-UQ) of the colon adenocarcinoma (COAD), stomach
358 adenocarcinoma (STAD) and Uterus Corpus Endometrium Carcinoma (UCEC) cohorts of The
359 Cancer Genome Atlas (TCGA) Research Network were downloaded via the GDC data portal
360 (<https://portal.gdc.cancer.gov>) on April 10th, 2019. Of these cohorts, mutation calls of TCGA's
361 final project, the PanCanAtlas, were downloaded from Synapse (syn7824274) on September
362 18th, 2017. These mutation calls were generated in a standardized pipeline across all samples,
363 resulting in a uniform dataset. Mismatch repair-deficiency status was obtained from Thorsson
364 *et al*³⁵ (TCGA Subtype = GI.HM-indel or UCEC.MSI).

365 **NICHE study sequencing data**

366 Raw RNA reads (FASTA files) of our recently published NICHE study¹⁰ (ClinicalTrials.gov:
367 [NCT03026140](https://clinicaltrials.gov/ct2/show/study/NCT03026140)) were generated as described in the original publication and aligned to the
368 human reference genome (GRCh38) with STAR software³⁶, version 2.7.7a, using default
369 settings. For gene expression quantification, we used the gencode.v35.annotation.gtf
370 annotation file. Somatic mutation data were obtained from DNA sequencing of pre-treatment
371 tumor biopsies and matched germline DNA, as described in the original publication¹⁰.

372 **Differential expression analysis**

373 Differential RNA expression of genes was tested in R using EdgeR³⁷, Limma³⁸ and Voom³⁹.
374 Raw read counts were filtered by removing lowly expressed genes. Normalization factors were
375 calculated using EdgeR, in order to transform the raw counts to log₂ counts per million reads
376 (CPM) and calculate residuals using Voom. Voom was then used to fit a smoothed curve to
377 the $\sqrt{(\text{residual standard deviation})}$ by average gene expression, which was then plotted for
378 visual inspection to confirm that the appropriate threshold was used for filtering of lowly
379 expressed genes (defined as the minimal amount of filtering necessary to overcome a dipping
380 mean-variance trend at low counts). Next, Limma was used to calculate differential expression
381 of genes based on a linear model fit, considering the smoothed curve for sample weights,
382 and empirical Bayes smoothing of standard errors. False discovery rates (FDRs) were
383 calculated by Benjamini-Hochberg correction of the obtained p-values.

384 *TCGA data*

385 Using TCGA data, we calculated differential expression between tumors with and without high
386 impact mutations in *B2M*, adjusting for tumor type and tumor mutational burden (TMB), using

387 the following design formula: expression ~ Primary_Site + TMB + B2M_status (+ intercept by
388 default), for which Primary_Site was a three-leveled factor (COAD, STAD, or UCEC), TMB
389 was a continuous variable (\log_{10} [exome-wide number of mutations]) and B2M_status was a
390 two-leveled factor (mutated, or wildtype).

391 *NICHE study data*

392 Using NICHE study data, we calculated differential expression between pre- and post-ICB
393 treatment. In order to respect the paired nature of these data, we used the following design
394 formula: expression ~ Patient + ICB (+ intercept by default), for which Patient was a factor for
395 each individual patient and ICB was a two-leveled factor (ICB-treated yes/no).

396 **Immune marker gene set expression analysis**

397 *TCGA data*

398 To utilize RNA-seq data in order to obtain a relative estimate of the infiltration of specific
399 immune cell types within tumors of TCGA, we summed the $\log_2(\text{FPKM-UQ}+1)$ expression of
400 genes that are specifically expressed in the immune cell types of interest. To this end, we used
401 the marker gene sets published by Danaher *et al.*¹⁹, and extended this by (i) the CD4 T cell
402 marker genes of Davoli *et al.*⁴⁰, (ii) a $\gamma\delta$ T cell receptor gene set (comprised of all genes whose
403 name starts with “TRDC”, “TRGC”, “TRDV”, “TRGV”, “TRDJ”, “TRGJ”), and (iii) a killer-cell Ig-
404 like receptor (KIR) gene set (comprised of all genes whose name starts with “KIR” and whose
405 name contains “DL” or “DS”. We excluded the gene set “NK CD56dim cells” of Danaher *et al.*
406 (comprising IL21R, KIR2DL3, KIR3DL1, and KIR3DL2) from our analyses, as three out of four
407 genes within this set were KIRs and hence this set showed high collinearity/redundancy to our
408 full KIR gene set. The gene set-specific expression values were Z-score transformed. For
409 TCGA-based analyses of MMR-d tumors, association of marker gene set expression with *B2M*
410 mutation status (high impact mutation yes/no) was calculated using ordinary least squares
411 linear regression, as implemented in the Python package Statsmodels
412 (<https://pypi.org/project/statsmodels/>), adjusting for tumor type and TMB as described above
413 for the TCGA differential expression analysis.

414 *NICHE study data*

415 Based on NICHE study data, we tested if expression of the $\gamma\delta$ T cell receptor gene set and
416 killer-cell Ig-like receptor (KIR) gene set were more strongly induced upon ICB-treatment in
417 *B2M^{MUT}* MMR-d tumors as compared to *B2M^{WT}* MMR-d tumors. First, we summed the
418 $\log_2(\text{reads per million}+1)$ expression of the genes within the two concerning gene sets for each
419 sample. Next, we fitted an ordinary least squares linear regression model (Statsmodels, see

420 above) that respects the paired nature of the data, using the following design formula:
421 expression \sim Patient + ICB + B2M + ICB:B2M + intercept, where Patient was a factor for each
422 individual patient, ICB was a two-leveled factor (ICB-treated yes/no), *B2M* was a two-leveled
423 factor ($B2M^{MUT}/B2M^{WT}$), and ICB:B2M was an interaction term between ICB and *B2M*, which
424 represents the statistic of interest (is ICB-based induction of expression of the two gene sets
425 significantly different between $B2M^{MUT}$ and $B2M^{WT}$ patients).

426 Hierarchical clustering

427 Hierarchical clustering of immune marker gene set expression profiles (Z-scores) of TCGA
428 cohorts was performed using the Python package Scipy⁴¹, with Euclidean distance as distance
429 metric and using the Ward variance minimization algorithm.

430 Patient samples

431 Primary colon cancer tissues were from 17 patients with colon cancer who underwent surgical
432 resection of their tumor at the Leiden University Medical Center (LUMC, the Netherlands)
433 ([Extended Data Table 1](#)). No patient with a previous history of inflammatory bowel disease
434 was included. This study was approved by the Medical Ethical Committee of the Leiden
435 University Medical Center (protocol P15.282), and patients provided written informed consent.
436 In addition, primary colon cancer tissues from 10 patients with colon cancer included in the
437 NICHE study ([NCT03026140](#))¹⁰ carried out at the Netherlands Cancer Institute (NKI, the
438 Netherlands) were used for this study. All specimens were anonymized and handled according
439 to the ethical guidelines described in the Code for Proper Secondary Use of Human Tissue in
440 the Netherlands of the Dutch Federation of Medical Scientific Societies.

441 Processing of colorectal cancer tissues

442 Details on the processing of colorectal tumor tissues have been described previously¹⁸. In
443 short, macroscopic sectioning from the lumen to the most invasive area of the tumor was
444 performed. Tissues were collected in IMDM+Glutamax medium (Gibco) complemented with
445 20% fetal calf serum (FCS) (Sigma-Aldrich), 1% pen/strep (Gibco) and fungizone (Gibco), and
446 0.1% ciprofloxacin (provided by apothecary LUMC) and gentamicin (Invitrogen), and
447 immediately cut into small fragments in a petri dish. Enzymatical digestion was performed with
448 1 mg/mL collagenase D (Roche Diagnostics) and 50 μ g/mL DNase I (Roche Diagnostics) in 5
449 mL of IMDM+Glutamax medium for 30 min at 37°C in gentleMACS C tubes (Miltenyi Biotec).
450 During and after incubation, cell suspensions were dissociated mechanically on the
451 gentleMACS Dissociator (Miltenyi Biotec). Cell suspensions were filtered through a 70- μ m cell
452 strainer (Corning), washed in IMDM+Glutamax medium with 20% FCS, 1% pen/strep, and

453 0.1% fungizone, and cell count and viability were determined with the Muse Count & Viability
454 Kit (Merck) on the Muse Cell Analyser (Merck). Based on the number of viable cells, cells in
455 IMDM+Glutamax medium were cryopreserved in liquid nitrogen until time of analysis
456 complemented 1:1 with 80% FCS and 20% dimethyl sulfoxide (DMSO) (Merck).

457 **Immunohistochemical detection of MMR, β 2m, and HLA class I proteins**

458 Tumor MMR status was determined by immunohistochemical detection of PMS2 (anti-PMS2
459 antibodies; clone EP51, DAKO) and MSH6 (anti-MSH6 antibodies; clone EPR3945, Abcam)
460 proteins⁴². MMR-deficiency was defined as the lack of expression of at least one of the MMR-
461 proteins in the presence of an internal positive control. Tumor β 2m status was determined by
462 immunohistochemical detection of β 2m (anti- β 2m antibodies; clone EP2978Y, Abcam).
463 Immunohistochemical detection of HLA class I expression on tumors was performed with
464 HCA2 and HC10 monoclonal antibodies (Nordic-MUbio), and classified as HLA class I
465 positive, weak, or loss as described previously⁶.

466 **Imaging mass cytometry staining and analysis**

467 Imaging mass cytometry (IMC) was performed on ICB-naïve colon cancer tissues (MMR-d) of
468 17 patients from the LUMC, of which four HLA class I-positive, eight HLA class I-defect, and
469 five β 2m-defect ([Extended Data Table 1](#)). In addition, IMC was performed on ICB-treated colon
470 cancer tissues (MMR-d) of ten patients from the NKI, of which five $B2M^{WT}$ and five $B2M^{MUT}$.
471 Antibody conjugation and immunodetection were performed following the methodology
472 published previously by Ijsselsteijn *et al.*⁴³. Four- μ m FFPE tissue were incubated with 41
473 antibodies in four steps. First, sections were incubated with anti-CD4 and anti-TCR δ overnight
474 at RT, which were subsequently detected using metal-conjugated secondary antibodies (goat
475 anti-rabbit IgG and goat anti-mouse IgG, respectively; Abcam). Second, sections were
476 incubated with 20 antibodies ([Extended Data Table 3](#)) for five hours at RT. Third, sections
477 were incubated overnight at 4°C with the remaining 19 antibodies ([Extended Data Table 3](#)).
478 Fourth, sections were incubated with 0.125 μ M Cell-ID intercalator-Ir (Fluidigm) to detect the
479 DNA, and stored dry until measurement. For each sample, six 1000x1000 μ m regions were
480 selected based on consecutive Haematoxylin and Eosin (H&E) stains and ablated using the
481 Hyperion Imaging system (Fluidigm). Data was acquired with the CyTOF Software (version
482 7.0) and exported with MCD Viewer (version 1.0.5). Data was normalised using semi-
483 automated background removal in ilastik⁴⁴, version 1.3.3, to control for variations in signal-to-
484 noise between FFPE sections as described previously⁴⁵. Thereafter, the phenotype data was
485 normalized at pixel level. Cell segmentation masks were created for all CD3- and/or CD7-
486 positive cells in ilastik and CellProfiler⁴⁶, version 2.2.0. In ImaCytE⁴⁷, version 1.1.4, cell

487 segmentation masks and normalized images were combined to generate single-cell FCS files
488 containing the relative frequency of positive pixels for each marker per cell. Cells forming
489 visual neighbourhoods in a t-distributed Stochastic Neighbour Embedding (t-SNE)⁴⁸
490 embedding in Cytosplore⁴⁹, version 2.3.0, were grouped and exported as separate FCS files.
491 The resulting subsets were imported back into ImaCyte and visualized on the segmentation
492 masks. Expression of immunomodulatory markers was determined as all cells with a relative
493 frequency of at least 0.2 positive pixels per cell. Differences in cells/mm² were calculated by
494 Mann-Whitney tests in Graphpad Prism (version 9.0.1).

495

496 **Sorting of $\gamma\delta$ T cells from colon cancers and single-cell RNA-sequencing**

497 scRNA-seq was performed on sorted $\gamma\delta$ T cells from colon cancers (MMR-d) of five patients
498 from the LUMC in the presence of hashtag oligo (HTOs) for sample ID and antibody-derived
499 tags (ADTs) for CD45RA and CD45RO protein expression by CITE-seq⁵⁰. Cells were thawed,
500 rest at 37°C in IMDM (Lonza)/20% FCS for 1h, followed by incubation with human Fc receptor
501 block (BioLegend) for 10 min at 4°C. Thereafter, cells were stained with cell surface antibodies
502 (1:50 anti-CD3-PE [clone SK7, BD Biosciences], 1:160 anti-CD45-PerCP-Cy5.5 [clone 2D1,
503 eBioscience], 1:200 anti-CD7-APC [clone 124-1D1, eBioscience], 1:60 anti-EPCAM-FITC
504 [clone HEA-125, Miltenyi], 1:80 anti-TCR $\gamma\delta$ -BV421 [clone 11F2, BD Biosciences], and a
505 1:1000 near-infrared viability dye [Life Technologies]), 1 μ g of TotalSeq-C anti-CD45RA (clone
506 HI100, BioLegend) and 1 μ g of anti-CD45RO (clone UCHL1, BioLegend) antibodies, and 0.5
507 μ g of a unique TotalSeq-C CD298/ β 2M hashtag antibody (clone LNH-94/2M2, BioLegend) for
508 each sample (n=5) for 30 min at 4°C. Cells were washed three times in FACS buffer (PBS
509 (Fresenius Kabi)/1% FCS) and kept cold and dark until cell sorting. Compensation was carried
510 out with CompBeads (BD Biosciences) and ArC reactive beads (Life Technologies). Single,
511 live CD45⁺ EPCAM⁻ CD3⁺ TCR $\gamma\delta$ ⁺ cells from five colorectal tumors (MMR-d) were sorted on
512 a FACS Aria III 4L (BD Biosciences). After sorting, the samples were pooled.

513 scRNA-seq libraries were prepared using the Chromium Single Cell 5' Reagent Kit v1
514 chemistry (10X Genomics) following the manufacturer's instructions. The construction of 5'
515 Gene Expression libraries allowed the identification of $\gamma\delta$ T cell subsets according to V δ and
516 V γ usage. Libraries were sequenced on a HiSeq X Ten using paired-end 2x150 bp sequencing
517 (Illumina). Reads were aligned to the human reference genome (GRCh38) and quantified
518 using Cell Ranger (version 3.1.0). Downstream analysis was performed using Seurat (version
519 3.1.5) according to the author's instructions⁵¹. Briefly, cells that had less than 200 detected
520 genes and genes that were expressed in less than six cells were excluded. The resulting 5669
521 cells were demultiplexed based on HTO enrichment using the MULTISEQDemux algorithm⁵².

522 Next, cells with a mitochondrial gene content greater than 10% and cells with outlying numbers
523 of expressed genes (>3000) were filtered out from the analysis, resulting in a final dataset of
524 4442 cells. Data were normalized using the 'LogNormalize' function from Seurat with scale
525 factor 10,000. Variable features were identified using the 'FindVariableFeatures' function from
526 Seurat returning 2,000 features. We then applied the 'RunFastMNN' function from
527 SeuratWrappers split by sample ID to adjust for potential batch-derived effects across
528 samples⁵³. Uniform manifold approximation (UMAP)⁵⁴ was used to visualize the cells in a two-
529 dimensional space, followed by the 'FindNeighbors' and 'FindClusters' functions from Seurat.
530 Data were scaled and heterogeneity associated with mitochondrial contamination was
531 regressed out. Cell clusters were identified by performing differentially expressed gene
532 analysis with the 'FindAllMarkers' function with min.pct and logfc.threshold at 0.25.
533 Percentage of *TRDV1* (V δ 1), *TRDV2* (V δ 2), or *TRDV3* (V δ 3) positive cells was determined as
534 the percentage of all cells with an expression level of >1, while <1 for the other TCR V δ chains.
535 CRC96, 134 and 167 had less than ten *TRDV3*⁺ cells, and were not included in the V δ 3
536 analysis. Transcripts of V δ 4 (*TRDV4*), V δ 5 (*TRDV5*), and V δ 8 (*TRDV8*) cells were not
537 detected. Percentage of *TRGV1* (V γ 1) – *TRGV11* (V γ 11) positive cells was determined as the
538 percentage of all cells with an expression level of >1, while <1 for the other TCR V γ chains.
539 Percentage of cells positive for a certain gene was determined as all cells with an expression
540 level of >1.

541 **Sorting of $\gamma\delta$ T cells from colon cancers and cell culturing**

542 $\gamma\delta$ T cells from colon cancers (MMR-d) of five patients from the LUMC were sorted for cell
543 culture. Cells were thawed and rest at 37°C in IMDM (Lonza)/10% nHS for 1h. Thereafter,
544 cells were incubated with human Fc receptor block (BioLegend) and stained with cell surface
545 antibodies (1:20 anti-CD3-Am Cyan [clone SK7, BD Biosciences], 1:80 anti-TCR $\gamma\delta$ -BV421
546 [clone 11F2, BD Biosciences], and 1:30 anti-PD-1-PE [clone MIH4, eBioscience] for 45 min at
547 4°C together with different additional antibodies for immunophenotyping (including 1:10 anti-
548 CD103-FITC [clone Ber-ACT8, BD Biosciences], 1:200 anti-CD38-PE-Cy7 [clone HIT2,
549 eBioscience], 1:60 anti-CD39-APC [clone A1, BioLegend], 1:20 anti-CD45RA-PE-Dazzle594
550 [clone HI100, Sony], 1:20 anti-CD45RO-PerCP-Cy5.5 [clone UCHL1, Sony], 1:40 anti-TCR $\alpha\beta$ -
551 PE-Cy7 [clone IP26, BioLegend], 1:50 anti-TCRV δ 1-FITC [clone TS8.2, Invitrogen], or 1:200
552 anti-TCRV δ 2-PerCP-Cy5.5 [clone B6, BioLegend]. A 1:1000 live/dead fixable near-infrared
553 viability dye (Life Technologies) was included in each staining. Cell were washed three times
554 in FACS buffer (PBS/1% FCS) and kept cold and dark until cell sorting. Compensation was
555 carried out with CompBeads (BD Biosciences) and ArC reactive beads (Life Technologies).
556 Single, live CD3⁺ TCR $\gamma\delta$ ⁺ PD-1⁺ and PD-1⁻ cells from five colorectal tumors (MMR-d) were

557 sorted on a FACS Aria III 4L (BD Biosciences). For CRC94 all $\gamma\delta$ T cells were sorted due to
558 the low number of PD-1⁺ cells. $\gamma\delta$ T cells were sorted in medium containing feeder cells
559 (1x10⁶/mL), PHA (1 μ g/mL; Thermo Fisher Scientific), IL-2 (1000 IU/mL; Novartis), IL-15 (10
560 ng/mL; R&D Systems), gentamicin (50 μ g/mL), and fungizone (0.5 μ g/mL). Sorted $\gamma\delta$ T cells
561 were expanded in the presence of 1000 IU/mL IL-2 and 10 ng/mL IL-15 for three-four weeks.
562 Purity and phenotype of $\gamma\delta$ T cells were assessed by flow cytometry. We obtained a >170,000-
563 fold increase in 3-4 weeks of expansion of $\gamma\delta$ T cells ([Extended Data Fig. 4e](#)).

564 **Immunophenotyping of expanded $\gamma\delta$ T cells by flow cytometry**

565 Expanded $\gamma\delta$ T cells from colon tumors were analyzed by flow cytometry for the expression of
566 TCR V δ chains, NKG2 receptors, NCRs, KIRs, tissue-residency/activation markers, cytotoxic
567 molecules, immune checkpoint molecules, cytokine receptors, and Fc receptors. Briefly, cells
568 were incubated with human Fc receptor block (BioLegend) and stained with cell surface
569 antibodies ([Extended Data Table 4](#)) for 45 min at 4°C, followed by three washing steps in
570 FACS buffer (PBS/1% FCS). Granzyme B and perforin were detected intracellularly using
571 Fixation Buffer and Intracellular Staining Permeabilization Wash Buffer (BioLegend).
572 Compensation was carried out with CompBeads (BD Biosciences) and ArC reactive beads
573 (Life Technologies). Cells were acquired on a FACS LSR Fortessa 4L (BD Biosciences)
574 running FACSDiva software version 9.0 (BD Biosciences). Data were analyzed with FlowJo
575 software version 10.6.1 (Tree Star Inc).

576 **Cell culture of cancer cell lines**

577 Human colorectal adenocarcinoma cell lines HCT-15 (MMR-d), LoVo (MMR-d), HT-29 (MMR-
578 p), SW403 (MMR-p), and SK-CO-1 (MMR-p) as well as HLA class I deficient human leukemia
579 cell line K-562 and Burkitt lymphoma cell line Daudi were used as targets for reactivity and
580 immune cell killing assays. The cell lines were authenticated by STR profiling and tested for
581 mycoplasma. HCT-15, LoVo, HT-29, K-562, and Daudi cells were maintained in RPMI
582 (Gibco)/10% FCS. SW403 and SK-CO-1 were maintained in DMEM/F12 (Gibco)/10% FCS.
583 All adherent cell lines were trypsinized before passaging.

584 **Organoid models and culture**

585 Tumor organoids were derived from MMR-d CRC tumor of two patients via resection from the
586 colon, tumor organoid 1, or peritoneal biopsy, tumor organoid 2 ([Extended Data Table 2](#)).
587 Establishment of the respective organoid lines from tumor material was performed as
588 previously reported^{55,56}. Briefly, tumor tissue was mechanically dissociated and digested with
589 1.5 mg/mL of collagenase II (Sigma-Aldrich), 10 μ g/mL of hyaluronidase type IV (Sigma-

590 Aldrich), and 10 μ M Y-27632 (Sigma-Aldrich). Cells were embedded in Cultrex® RGF BME
591 Type 2 (cat no. 3533-005-02, R&D systems) and placed in a 37°C incubator for 20 min. Human
592 CRC organoids medium is composed of Ad-DF+++ (Advanced DMEM/F12 (GIBCO)
593 supplemented with 2 mM Ultraglutamine I (Lonza), 10 mM HEPES (GIBCO), and 100/100
594 U/mL Penicillin/Streptomycin (GIBCO), 10% Noggin-conditioned medium, 20% R-spondin1-
595 conditioned medium, 1x B27 supplement without vitamin A (GIBCO), 1.25 mM N-
596 acetylcysteine (Sigma-Aldrich), 10 mM nicotinamide (Sigma-Aldrich), 50 ng/mL human
597 recombinant EGF (Peprotech), 500 nM A83-01 (Tocris), 3 μ M SB202190 (Cayman Chemicals)
598 and 10 nM prostaglandin E2 (Cayman Chemicals). Organoids were passaged depending on
599 growth every 1–2 weeks by incubating in TrypLE Express (Gibco) for 5–10 min followed by
600 embedding in BME. Organoids were authenticated by SNP array or STR profile and regularly
601 tested for Mycoplasma using Mycoplasma PCR43 and the MycoAlert Mycoplasma Detection
602 Kit (cat no. LT07-318). In the first two weeks of organoid culture, 1x Primocin (Invivogen) was
603 added to prevent microbial contamination. Procedures performed with patient specimens were
604 approved by the Medical Ethical Committee of the Netherlands Cancer Institute – Antoni van
605 Leeuwenhoek hospital (study NL48824.031.14) and written informed consent was obtained
606 from all patients. Mismatch repair status was assessed by standard protocol for the Ventana
607 automated immunostainer for MLH1 clone M1 (Roche), MSH2 clone G219-1129 (Roche),
608 MSH6 clone EP49 (Abcam) and PMS2 clone EP51 (Agilent Technologies). The *B2M*^{KO} tumor
609 organoid lines were generated by using sgRNA targeting *B2M*
610 (GGCCGAGATGTCTCGCTCCG), cloned into LentiCRISPR v2 plasmid. The virus was
611 produced by standard method.

612 **Screening of cancer cell lines and tumor organoids by flow cytometry**

613 The cancer cell lines used in the reactivity and killing assays were screened for the expression
614 of HLA class I molecules, NKG2D ligands, DNAM-1 ligands, and butyrophilin by flow
615 cytometry. Briefly, cells were incubated with human Fc receptor block (BioLegend) and stained
616 with the different cell surface antibodies (1:10 anti-CD112-PE [clone R2.525, BD Biosciences],
617 1:10 anti-CD155-PE [clone 300907, R&D Systems], 1:50 anti-CD277/BTN3A1-PE [clone
618 BT3.1, Miltenyi], 1:100 anti-HLA-A,B,C-FITC [clone W6/32, eBioscience], 1:20 anti-HLA-E-
619 BV421 [clone 3D12, BioLegend], 1:20 anti-HLA-G-APC [clone 87G, BioLegend], 1:300 anti-
620 MICA/B-PE [clone 6D4, BioLegend], 1:10 anti-ULBP1-PE [clone 170818, R&D Systems], 1:20
621 anti-ULBP2/5/6-PE [clone 165903, R&D Systems], 1:20 anti-ULBP3-PE [clone 166510, R&D
622 Systems], or 1:20 anti-ULBP4-PE [clone 709116, R&D Systems] for 45 min at 4°C. A 1:1000
623 live/dead fixable near-infrared viability dye (Life Technologies) was included in each staining.
624 Cells were washed three times in FACS buffer (PBS/1% FCS). Compensation was carried out

625 with CompBeads (BD Biosciences) and ArC reactive beads (Life Technologies). Cells were
626 acquired on a FACS Canto II 3L or FACS LSR Fortessa 4L (BD Biosciences) running
627 FACSDiva software version 9.0 (BD Biosciences). Isotype or FMO controls were included to
628 determine the percentage of positive cancer cells. Data were analyzed with FlowJo software
629 version 10.6.1 (Tree Star Inc).

630 For organoid surface staining, tumor organoids were dissociated into single cells using
631 TrypLE Express (Gibco) washed twice in cold FACS buffer (PBS, 5 mM EDTA, 1% bovine
632 serum antigen) and stained with either 1:20 anti-HLA-A,B,C-PE (clone W6/32, BD
633 Biosciences), 1:100 anti- β 2m-FITC (clone 2M2, BioLegend), 1:200 anti-PD-L1 (clone MIH1,
634 eBioscience) and 1:2000 near-infrared (NIR) viability dye (Life Technologies) or isotype
635 controls (FITC, PE or APC) mouse IgG1 kappa (BD Biosciences). For NKG2D ligand
636 expression analysis cells were stained with 1:300 anti-MICA/MICB, 1:10 anti-ULBP1, 1:20
637 anti-ULBP2/5/6, 1:20 anti-ULBP3, 1:20 anti-ULBP4, and 1:2000 near-infrared (NIR) viability
638 dye (Life Technologies). Tumor cells were incubated for 30 min at 4°C in the dark and washed
639 twice in FACS buffer. All samples were recorded at a Becton Dickinson Fortessa.

640 **Reactivity assay $\gamma\delta$ T cells**

641 Reactivity of $\gamma\delta$ T cells to the different cancer cell lines was assessed by a co-culture reactivity
642 assay. $\gamma\delta$ T cells were thawed and cultured in IMDM+Glutamax (Gibco)/8% nHS medium with
643 pen (100 IU/mL)/strep (100 μ g/mL) in the presence of low-dose IL-2 (25 IU/mL) and IL-15 (5
644 ng/mL) overnight at 37°C. Cancer cell lines were counted, adjusted to a concentration of
645 0.5×10^5 cells/mL in IMDM+Glutamax/10% FCS medium with pen (100 IU/mL)/strep (100
646 μ g/mL), and seeded (100 μ L/well) in coated 96-well flat-bottom microplates (Greiner CellStar)
647 (for 5,000 cells/well) overnight at 37°C. The next day, $\gamma\delta$ T cells were harvested, counted, and
648 adjusted to a concentration of 1.2×10^6 cells/mL in IMDM+Glutamax/10% FCS medium. The $\gamma\delta$
649 T cells were added in 50 μ L (for 60,000 cells/well) and co-cultured (12:1 E:T ratio) at 37°C for
650 18h in biological triplicates. Medium (without cancer cells) was used as negative control and
651 PMA (20 ng/mL)/ionomycin (1 μ g/mL) as positive control. After co-culture, the supernatant
652 was harvested to detect IFN γ secretion by ELISA (Mabtech) following the manufacturer's
653 instructions. Additionally, cells were harvested, incubated with human Fc receptor block
654 (BioLegend), and stained with cell surface antibodies (1:100 anti-CD137-APC [clone 4B4-1,
655 BD Biosciences], 1:150 anti-CD226/DNAM-1-BV510 [clone DX11, BD Biosciences], 1:400
656 anti-CD3-AF700 [clone UCHT1, BD Biosciences], 1:80 anti-CD39-APC [clone A1, BioLegend],
657 1:10 anti-CD40L-PE [clone TRAP1, BD Biosciences] or 1:30 anti-PD-1-PE [clone MIH4,
658 eBioscience], 1:40 anti-TCR $\gamma\delta$ -BV650 [clone 11F2, BD Biosciences], 1:300 anti-NKG2D-PE-

659 Cy7 [clone 1D11, BD Biosciences], and 1:20 anti-OX40-FITC [clone ACT35, BioLegend] for
660 45 min at 4°C. A 1:1000 live/dead fixable near-infrared viability dye (Life Technologies) was
661 included in each staining. Cells were washed three times in FACS buffer (PBS/1% FCS).
662 Compensation was carried out with CompBeads (BD Biosciences) and ArC reactive beads
663 (Life Technologies). Cells were acquired on a FACS LSR Fortessa X-20 4L (BD Biosciences)
664 running FACSDiva software version 9.0 (BD Biosciences). Data were analyzed with FlowJo
665 software version 10.6.1 (Tree Star Inc). All data are representative of at least two independent
666 experiments.

667 **Immune cell killing assay $\gamma\delta$ T cells**

668 Killing of the different cancer cell lines by $\gamma\delta$ T cells was visualized and quantified by a co-
669 culture immune cell killing assay using the IncuCyte S3 Live-Cell Analysis System (Essen
670 Bioscience). HCT-15, LoVo, and HT-29 cells were transduced with IncuCyte NucLight Red
671 Lentivirus Reagent (EF-1 α , Puro; Essen BioScience) providing a nuclear-restricted expression
672 of a red (mKate2) fluorescent protein. In short, HCT-15, LoVo and HT-29 were seeded,
673 transduced according to the manufacturer's instructions, and stable cell populations were
674 generated using puromycin selection. Cancer cell lines were counted, adjusted to a
675 concentration of 1×10^5 cells/mL in IMDM+Glutamax/10% FCS medium with pen (100
676 IU/mL)/strep (100 μ g/mL), and seeded (100 μ L/well) in 96-well flat-bottom clear microplates
677 (Greiner CellStar) (for 10,000 cells/well). The target cell plate was placed in the IncuCyte
678 system at 37°C to monitor for cell confluency for 3 days. On day 2, $\gamma\delta$ T cells were thawed and
679 cultured in IMDM+Glutamax/8% nHS medium with pen (100 IU/mL)/strep (100 μ g/mL) in the
680 presence of low-dose IL-2 (25 IU/mL) and IL-15 (5 ng/mL) overnight at 37°C. The next day, $\gamma\delta$
681 T cells were harvested, counted, and adjusted to a concentration of 7.2×10^5 cells/mL in
682 IMDM+Glutamax/10% FCS medium. After aspiration of the medium of the target cell plate,
683 100 μ L of new medium containing 3.75 μ M IncuCyte Caspase-3/7 Green Apoptosis Reagent
684 (Essen BioScience) (1.5x final assay concentration of 2.5 μ M) was added together with 50 μ L
685 of $\gamma\delta$ T cells (for 36,000 cells/well). They were co-cultured (4:1 E:T ratio) in the IncuCyte
686 system at 37°C in biological duplicates. Cancer cells alone and cancer cells alone with
687 Caspase-3/7 were used as negative controls. Images (2 images/well) were captured every
688 hour at 20x magnification with the phase, green, and red channels for up to 4 days.

689 Analysis was performed in the IncuCyte software (version 2020B) for each cancer cell
690 line separately. The following analysis definitions were applied: 1) for HCT-15 cells in the
691 phase channel a minimum area of 200 μ m², in the green channel a threshold of 2 GCU, and
692 in the red channel a threshold of 2 RCU, 2) for LoVo and HT-29 cells in the phase channel a

693 minimum area of 200 μm^2 , in the green channel a threshold of 4 GCU, and in the red channel
694 a threshold of 2 RCU. Cancer cell apoptosis was then quantified in the IncuCyte software by
695 counting the total number of Green + Red objects per image normalized (by division) to the
696 total number of Red objects per image after 12h co-culture and displayed as a percentage
697 (mean \pm SEM) of two wells with two images/well.

698 **Tumor organoid recognition assay**

699 For evaluation of tumor reactivity towards *B2M^{WT}* and *B2M^{KO}* organoids and NKG2D ligand
700 blocking conditions, tumor organoids and $\gamma\delta$ T cells were prepared as described
701 previously.^{10,55,56} Two days prior to the experiment organoids were isolated from BME by
702 incubation in 2 mg/mL type II dispase (Sigma-Aldrich) for 15 min before addition of 5 mM
703 ethylenediaminetetraacetic acid (EDTA) and washed with PBS before resuspended in CRC
704 organoid medium with 10 μM Y-27632 (Sigma-Aldrich). Organoids were stimulated with 200
705 ng/mL IFN γ (Peprotech) 24 hours before the experiment. For the recognition assay and intra-
706 cellular staining tumor organoids were dissociated into single cells and plated in anti-CD28
707 (clone CD28.2 eBioscience) coated 96-well U-bottom plates with $\gamma\delta$ T cells at a 1:1
708 target:effector ratio in the presence of 20 $\mu\text{g}/\text{mL}$ anti-PD-1 (Merus). As positive control $\gamma\delta$ T
709 cells were stimulated with 50 ng/mL of phorbol 12-myristate 13 -acetate (Sigma-Aldrich) and
710 1 $\mu\text{g}/\text{mL}$ of ionomycin (Sigma-Aldrich). After 1h of incubation at 37°C, GolgiSTOP (BD
711 Biosciences, 1:1500) and GolgiPlug (BD Biosciences, 1:1000) were added. After 4h of
712 incubation at 37°C, $\gamma\delta$ T cells were washed twice in cold FACS buffer (PBS, 5 mM EDTA, 1%
713 bovine serum antigen) and stained with 1:20 anti-CD3-PerCP-Cy5.5 (BD Biosciences), 1:20
714 anti-TCR $\gamma\delta$ -PE (BD Bioscience), 1:20 anti-CD4-FITC (BD Bioscience) (not added in
715 experiments with NKG2D ligand blocking), 1:200 anti-CD8-BV421 (BD Biosciences) and
716 1:2000 near-infrared (NIR) viability dye (Life Technologies) for 30 min at 4°C. Cells were
717 washed, fixed and stained with 1:40 anti-IFN γ -APC (BD Biosciences) for 30 min at 4°C, using
718 the Cytotfix/Cytoperm Kit (BD Biosciences). After two washing steps, cells were resuspended
719 in FACS buffer and recorded at a BD LSRFortessa™ Cell Analyzer SORP flow cytometer with
720 FACSDiVa 8.0.2 (BD Biosciences) software.

721 **Blocking experiments with cancer cell lines and tumor organoids**

722 Reactivity of and killing by the $\gamma\delta$ T cells was examined in the presence of different blocking
723 antibodies to investigate which receptor-ligand interactions are involved. For DNAM-1
724 blocking, $\gamma\delta$ T cells were incubated with 3 $\mu\text{g}/\text{mL}$ purified anti-DNAM-1 (clone DX11, BD
725 Biosciences) for 1h at 37°C. For $\gamma\delta$ TCR blocking, $\gamma\delta$ T cells were incubated with 3 $\mu\text{g}/\text{mL}$
726 purified anti-TCR $\gamma\delta$ (clone 5A6.E9, Invitrogen) for 1h at 37°C, of which the clone we used was

727 tested to be best for use in $\gamma\delta$ TCR blocking assays⁵⁷. NKG2D ligands were blocked on the
728 cancer cell lines and single cells of tumor organoids by incubating the target cells with 12
729 $\mu\text{g}/\text{mL}$ anti-MICA/B (clone 6D4, BioLegend), 1 $\mu\text{g}/\text{mL}$ anti-ULBP1 (clone 170818, R&D
730 Systems), 3 $\mu\text{g}/\text{mL}$ anti-ULBP2/5/6 (clone 165903, R&D Systems), and 6 $\mu\text{g}/\text{mL}$ anti-ULBP3
731 (clone 166510, R&D Systems) for 1h at 37°C prior to plating with $\gamma\delta$ T cells. After incubation
732 with the blocking antibodies, the $\gamma\delta$ T cells were added to cancer cell lines HCT-15, LoVo, and
733 HT-29 as described above with a minimum of two biological replicates per blocking condition.
734 For organoid experiments, 1:50 anti-CD107a-FITC (clone H4A3, BioLegend) was added
735 during incubation.

736 As a control for Fc-mediated antibody effector functions, $\gamma\delta$ T cells alone were
737 incubated with the blocking antibodies in the presence of 2.5 μM IncuCyte Caspase-3/7 Green
738 Apoptosis Reagent (Essen BioScience) in the IncuCyte system at 37°C, and the number of
739 apoptotic $\gamma\delta$ T cells was quantified over time.

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803

804 **Index of supplemental information**

805 **Extended Data Fig. 1.** Association of *B2M* mutation status with expression of $\alpha\beta$ T cell
806 receptors.

807 **Extended Data Fig. 2.** Characterization of $\gamma\delta$ T cells from MMR-d colon cancers by scRNA-
808 seq.

809 **Extended Data Fig. 3.** Distinct clusters of $\gamma\delta$ T cells from MMR-d colon cancers by scRNA-
810 seq.

811 **Extended Data Fig. 4.** Sorting of PD-1⁺ and PD-1⁻ $\gamma\delta$ T cells from MMR-d colon cancers by
812 FACS and their TCR V δ chain usage.

813 **Extended Data Fig. 5.** Reactivity of $\gamma\delta$ T cells from MMR-d colon cancers towards cancer
814 cell lines.

815 **Extended Data Fig. 6.** Surface expression of activation markers and secretion of IFN γ upon
816 co-culture of $\gamma\delta$ T cells from MMR-d colon cancers with cancer cell lines.

817 **Extended Data Fig. 7.** Tumor organoid characterization and reactivity assay readout.

818 **Extended Data Fig. 8.** Surface expression of activating receptor NKG2D by PD-1⁺ $\gamma\delta$ T cells
819 from MMR-d colon cancers upon co-culture with cancer cell lines.

820 **Extended Data Fig. 9.** Killing of cancer cell lines by PD-1⁺ $\gamma\delta$ T cells from MMR-d colon
821 cancers in the presence NKG2D ligand, DNAM-1, or $\gamma\delta$ TCR blocking.

822 **Extended Data Fig. 10.** Reactivity towards cancer cell lines by PD-1⁺ $\gamma\delta$ T cells from MMR-d
823 colon cancers in the presence of NKG2D ligand blocking.

824 **Extended Data Fig. 11.** Distribution of immune cell populations in *B2M*-wildtype and *B2M*-
825 mutant colon cancers upon ICB by imaging mass cytometry.

826

827 **Extended Data Table 1.** Characteristics of clinical samples from 17 patients with MMR-d
828 colon cancer.

829 **Extended Data Table 2.** Characteristics of patient-derived organoids from MMR-d colorectal
830 cancer.

831 **Extended Data Table 3.** Antibodies used for imaging mass cytometry of colon cancers.

832 **Extended Data Table 4.** Antibodies used for immunophenotyping of $\gamma\delta$ T cells by flow
833 cytometry.

834

835 **Movie 1.** Killing of HCT-15 cells by $\gamma\delta$ T cells (V δ 1⁺) from a MMR-d colon cancer.

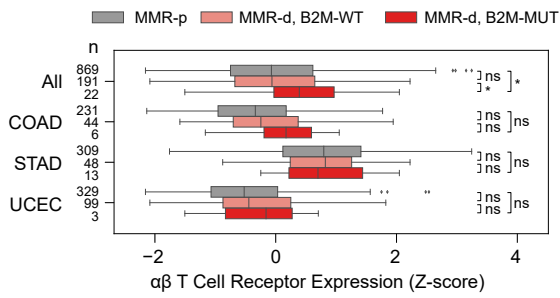
836 **Movie 2.** Killing of HCT-15 cells by PD-1⁺ (V δ 1⁺) as compared to PD-1⁻ (V δ 2⁺) $\gamma\delta$ T cells
837 from a MMR-d colon cancer.

838

839 **Abbreviations**

- 840 B2M/ β 2m, β 2-microglobulin
- 841 B2M^{KO}, β 2-microglobulin-knockout
- 842 B2M^{MUT}, β 2-microglobulin-mutant
- 843 B2M^{WT}, β 2-microglobulin-wildtype
- 844 BTN, butyrophilin
- 845 CML, chronic myelogenous leukemia
- 846 COAD, colon adenocarcinoma
- 847 CRC, colorectal cancer
- 848 ICB, immune checkpoint blockade
- 849 ICS, intra-cellular staining
- 850 KIR, killer-cell immunoglobulin-like receptor
- 851 MMR-d, mismatch repair-deficient
- 852 MMR-p, mismatch repair-proficient
- 853 NCR, natural cytotoxicity receptor
- 854 PDTO, patient-derived tumor organoid
- 855 scRNA-seq, single-cell RNA-sequencing
- 856 STAD, stomach adenocarcinoma
- 857 UCEC, uterus corpus endometrium carcinoma
- 858 UMAP, uniform manifold approximation and projection

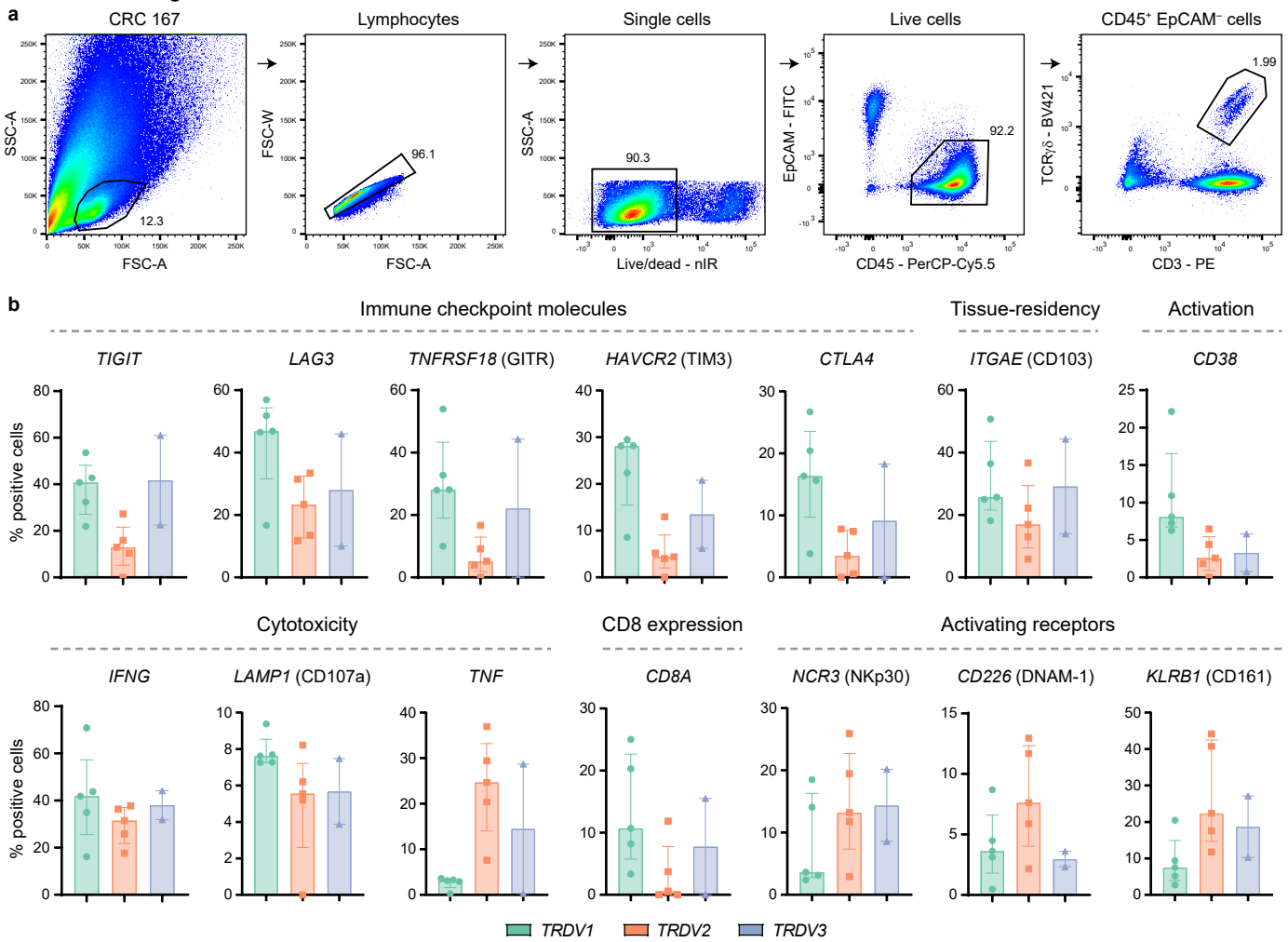
Extended Data Fig. 1



Extended Data Fig. 1. Association of *B2M* mutation status with expression of $\alpha\beta$ T cell receptors.

Boxplot showing the RNA expression of $\alpha\beta$ T cell receptors in MMR-p (gray), MMR-d *B2M*^{WT} (pink), and MMR-d *B2M*^{MUT} (high impact; red) cancers. Results are obtained on the TCGA COAD, STAD and UCEC cohorts, and are shown for all cohorts combined (All), and for each cohort separately. Boxes, whiskers, and dots indicate quartiles, 1.5 interquartile ranges, and outliers, respectively. P-values were calculated by Wilcoxon rank sum test. * P<0.05.

Extended Data Fig. 2

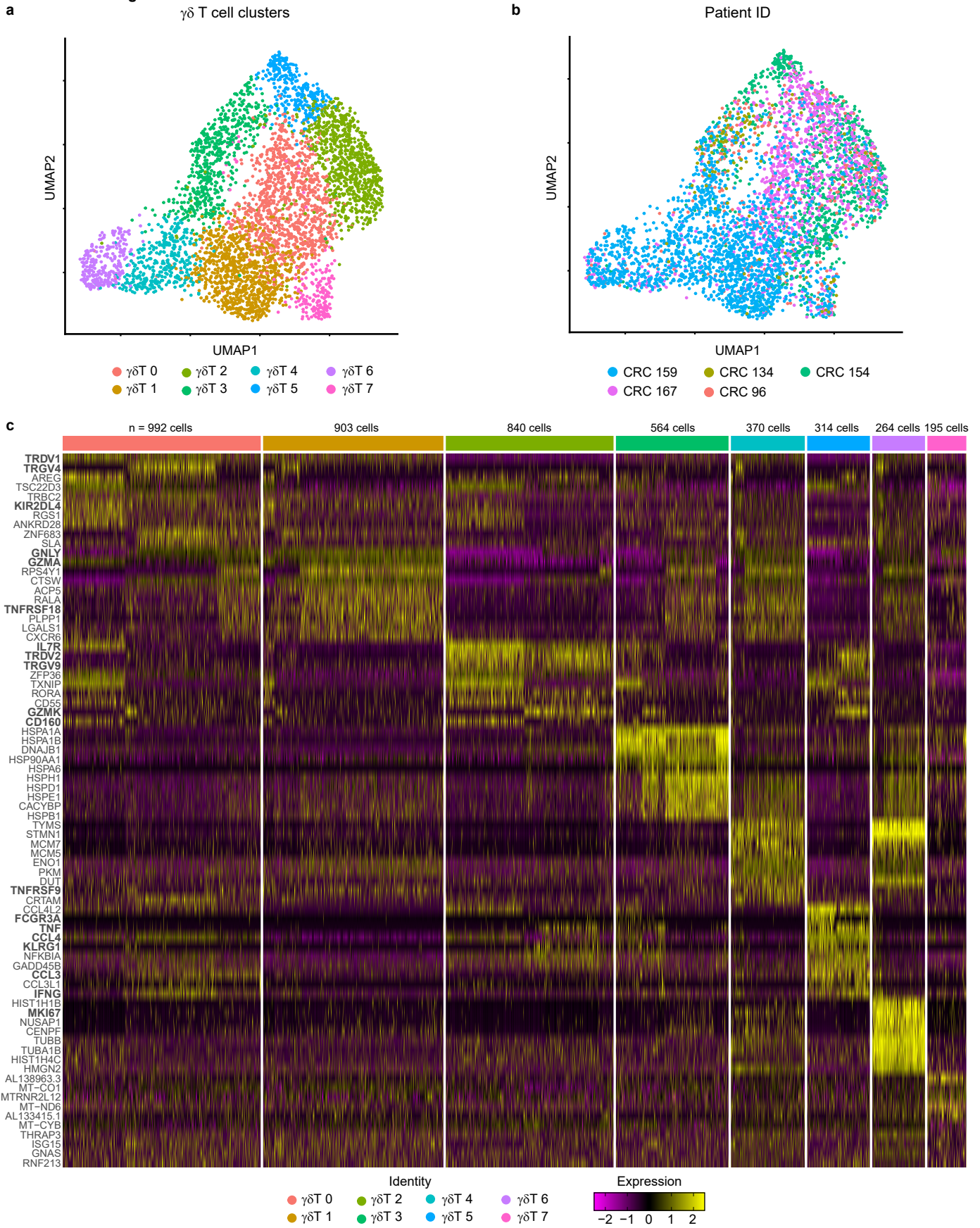


Extended Data Fig. 2. Characterization of $\gamma\delta$ T cells from MMR-d colon cancers by single-cell RNA-sequencing.

a. FACS gating strategy for single, live CD45⁺ EpCAM⁻ CD3⁺ TCR $\gamma\delta$ ⁺ cells of a representative MMR-d colon cancer sample showing sequential gates with percentages.

b. Frequencies of positive cells for selected genes across V δ 1 (n=1927), V δ 2 (n=860), and V δ 3 (n=506) cells as percentage of total $\gamma\delta$ T cells from each MMR-d colon tumor (n=5) analyzed by single-cell RNA-sequencing. V δ 3 cells were present in two out of five colon cancers. Bars indicate median \pm IQR. Each dot represents an individual sample.

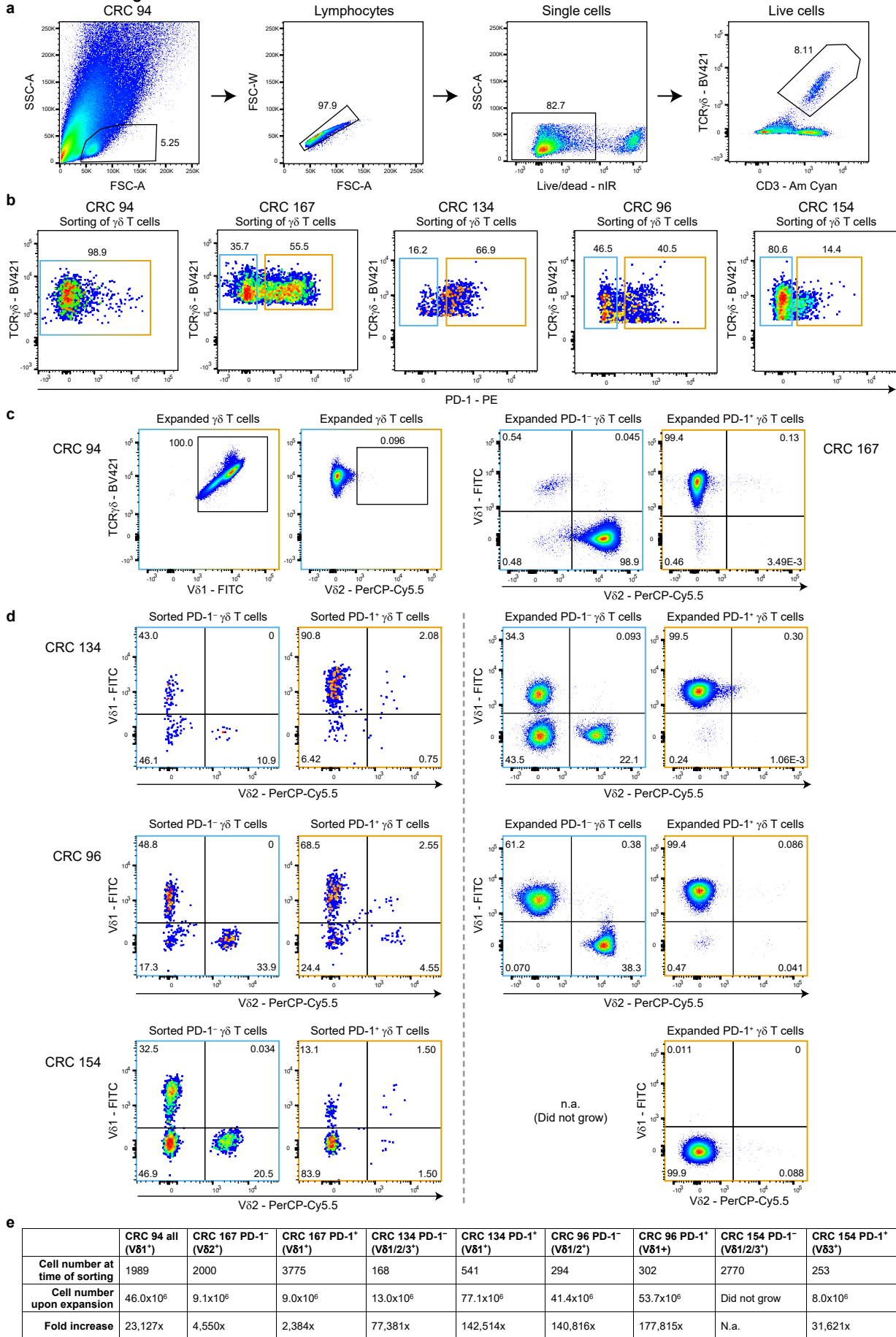
Extended Data Fig. 3



Extended Data Fig. 3. Distinct clusters of $\gamma\delta$ T cells from MMR-d colon cancers by single-cell RNA-sequencing.

- a.** UMAP embedding showing $\gamma\delta$ T cells (n=4442) isolated from MMR-d colon cancers (n=5) analyzed by single-cell RNA-sequencing. Colors represent the functionally different $\gamma\delta$ T cell clusters identified by graph-based clustering and non-linear dimensional reduction. Each dot represents a single cell.
- b.** UMAP embedding of (**a**) colored by patient ID. Each dot represents a single cell.
- c.** Heatmap showing the normalized single-cell gene expression value (z-score, purple-to-yellow scale) for the top 10 differentially expressed genes in each identified $\gamma\delta$ T cell cluster.

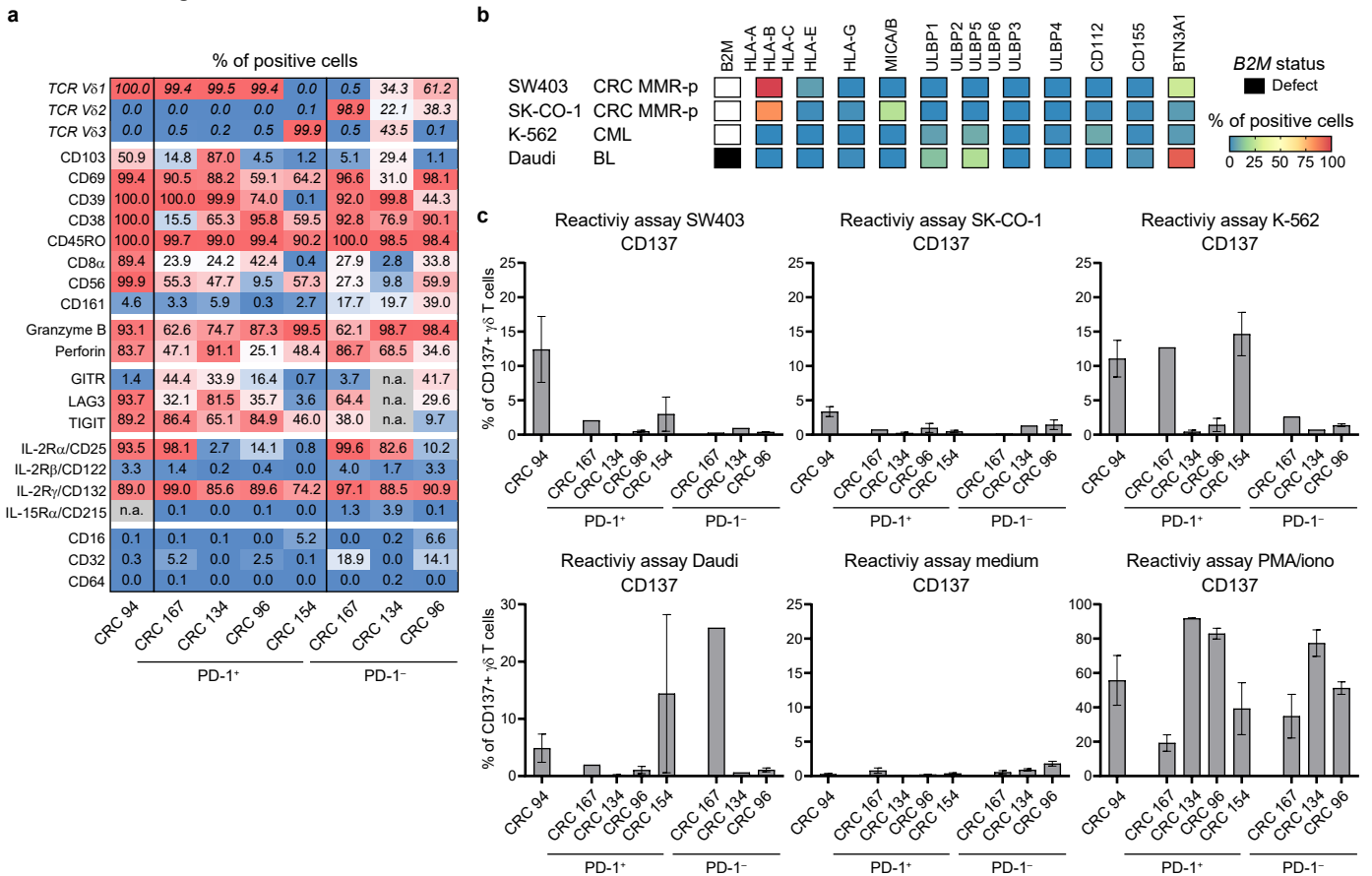
Extended Data Fig. 4



Extended Data Fig. 4. Sorting of PD-1⁺ and PD-1⁻ $\gamma\delta$ T cells from MMR-d colon cancers by FACS and their TCR V δ chain usage.

- a.** FACS gating strategy for single, live CD3⁺ TCR $\gamma\delta$ ⁺ cells of a representative MMR-d colon cancer sample showing sequential gates with percentages.
- b.** Sorting of all $\gamma\delta$ T cells from CRC94 (due to the low number of PD-1⁺ cells), and of PD-1⁻ (blue squares) and PD-1⁺ (orange squares) $\gamma\delta$ T cells from CRC167, CRC134, CRC96, and CRC154. Each dot is a single cell.
- c.** TCR V δ chain usage after expansion of $\gamma\delta$ T cells from CRC94 and CRC167. Each dot is a single cell.
- d.** TCR V δ chain usage at the time of sorting (left panel) as well as after expansion of $\gamma\delta$ T cells from CRC134, CRC96 and CRC154 (right panel). From CRC154, the PD-1⁻ $\gamma\delta$ T cells did not expand in culture. Each dot is a single cell.
- e.** Table showing the number of $\gamma\delta$ T cells isolated from colon cancers at the time of sorting versus 3-4 weeks after expansion, and the fold increase thereof.

Extended Data Fig. 5



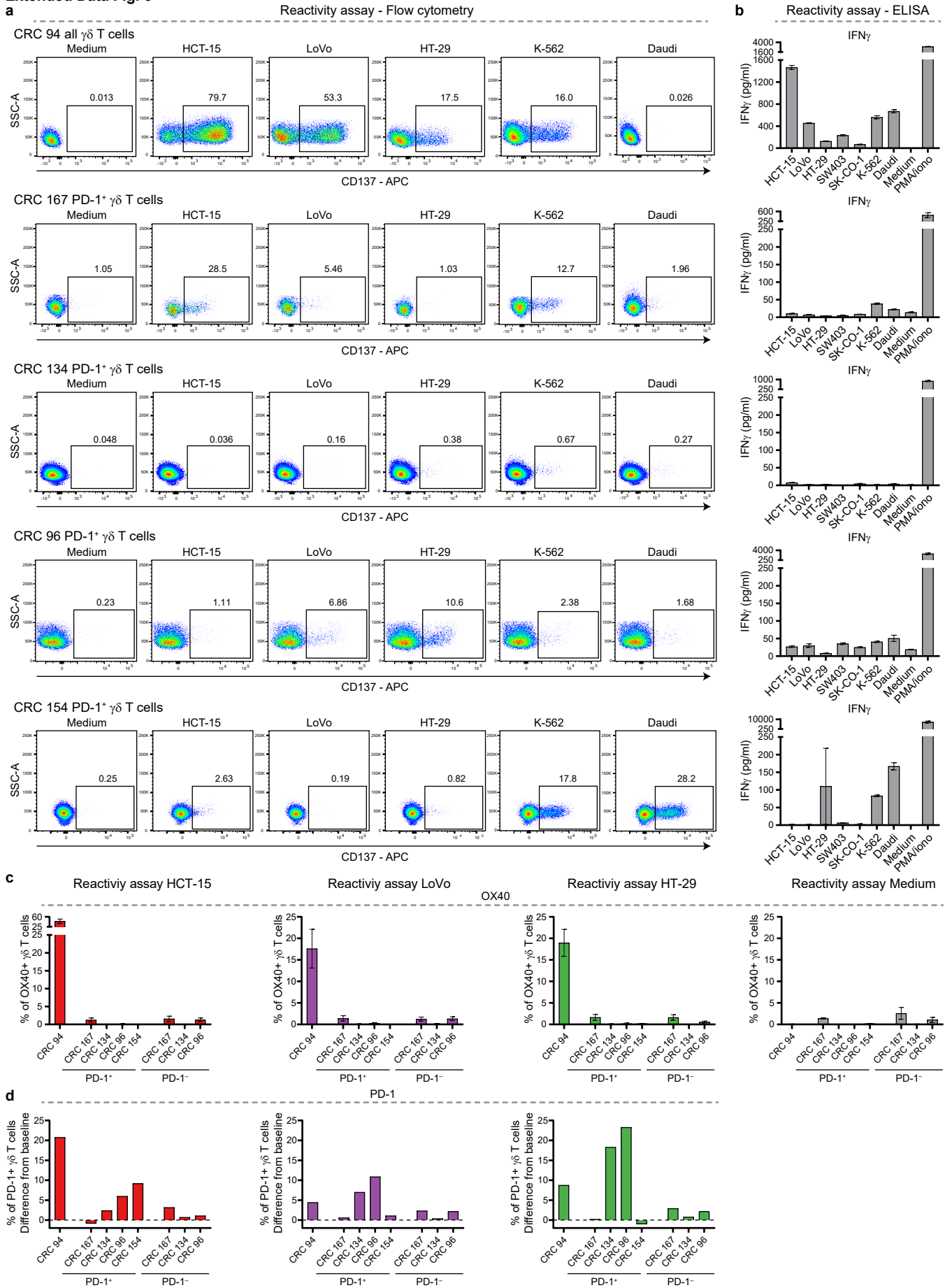
Extended Data Fig. 5. Reactivity of $\gamma\delta$ T cells from MMR-d colon cancers towards cancer cell lines.

a. Table showing the percentage of positive cells for different TCR V δ chains (as in Fig. 3a), tissue-residency/activation markers, cytotoxic molecules, immune checkpoint molecules, cytokine receptors, and Fc receptors on expanded PD-1⁺ and PD-1⁻ $\gamma\delta$ T cells from MMR-d colon cancers (n=5) as percentage of total $\gamma\delta$ T cells.

b. Heatmap showing the B2M mutational status and surface expression of HLA class I, NKG2D ligands, DNAM-1 ligands, and butyrophilin on SW403, SK-CO-1, K-562, and Daudi cells.

c. Bar plots showing the percentage of CD137-positive $\gamma\delta$ T cells after 18h co-culture of PD-1⁺ and PD-1⁻ $\gamma\delta$ T cells from MMR-d colon cancers (n=5) with SW403, SK-CO-1, K-562, and Daudi cells. Medium was used as negative control and PMA/ionomycin as positive control. Bars indicate mean \pm SEM. Data from two independent experiments (CRC94, CRC134, CRC154, CRC96), depending on availability of $\gamma\delta$ T cells.

Extended Data Fig. 6



Extended Data Fig. 6. Surface expression of activation markers and secretion of IFN γ upon co-culture of $\gamma\delta$ T cells from MMR-d colon cancers with cancer cell lines.

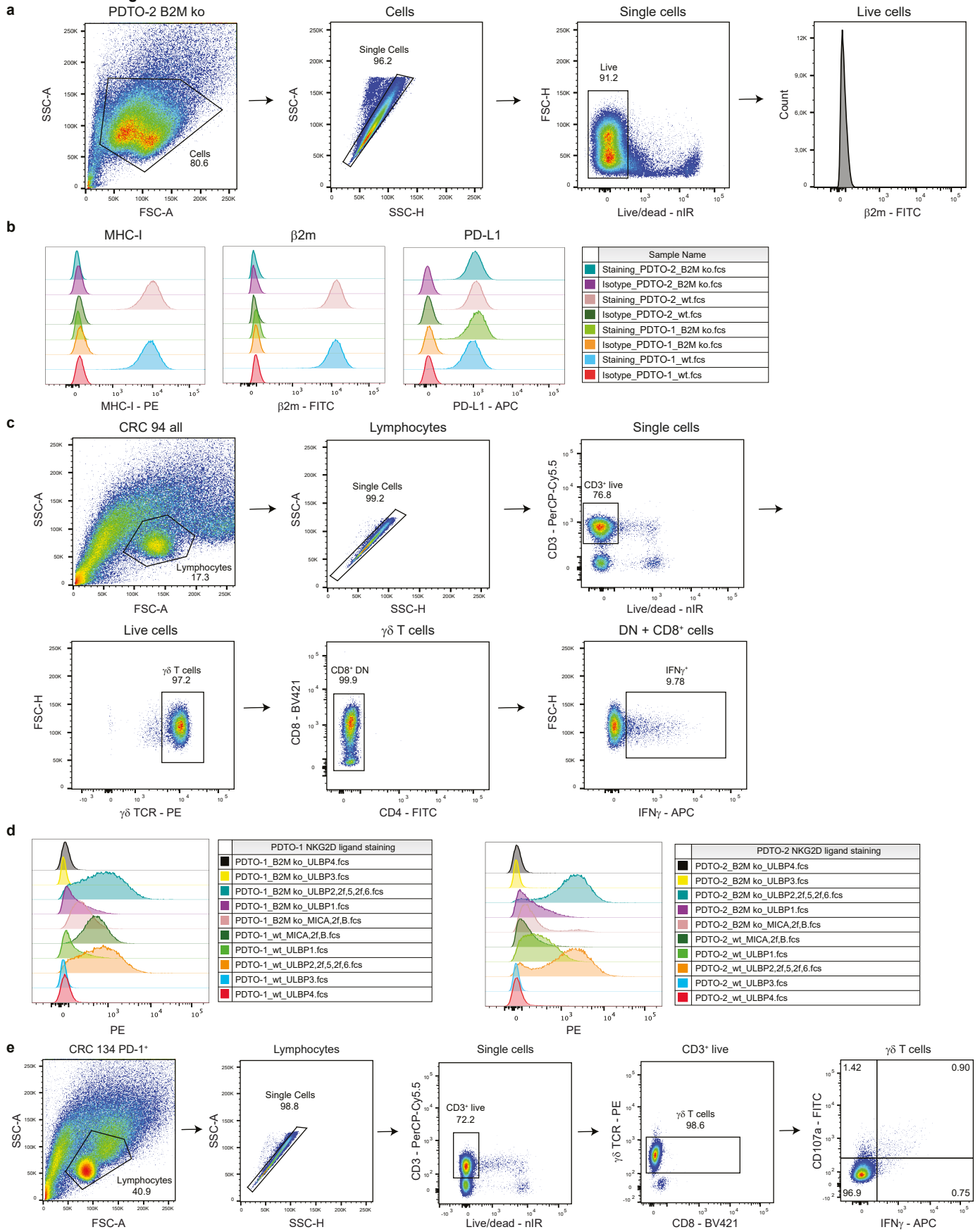
a. Flow cytometry plots showing the expression of CD137 on PD-1⁺ $\gamma\delta$ T cells after 18h co-culture with HCT-15, LoVo, HT-29, K-562, and Daudi cells as compared to medium only. Gates indicate percentage of positive $\gamma\delta$ T cells.

b. Bar plots showing the presence of IFN γ in the supernatant after 18h co-culture of PD-1⁺ $\gamma\delta$ T cells with the cancer cell lines. Medium as negative control and PMA/ionomycin as positive control are included. Bars indicate mean \pm SEM of triplicates.

c. Bar plots showing the percentage of OX40-positive $\gamma\delta$ T cells after 18h co-culture of PD-1⁺ and PD-1⁻ $\gamma\delta$ T cells from MMR-d colon cancers (n=5) with HCT-15, LoVo, and HT-29 cells. Bars indicate mean \pm SEM. Data from four (CRC94), three (CRC167, CRC96), or two (CRC134, CRC154) independent experiments, depending on availability of $\gamma\delta$ T cells.

d. Bar plots showing the expression of PD-1 on $\gamma\delta$ T cells as difference from baseline (medium) condition after 18h co-culture of PD-1⁺ and PD-1⁻ $\gamma\delta$ T cells from MMR-d colon cancers (n=5) with HCT-15, LoVo, and HT-29 cells.

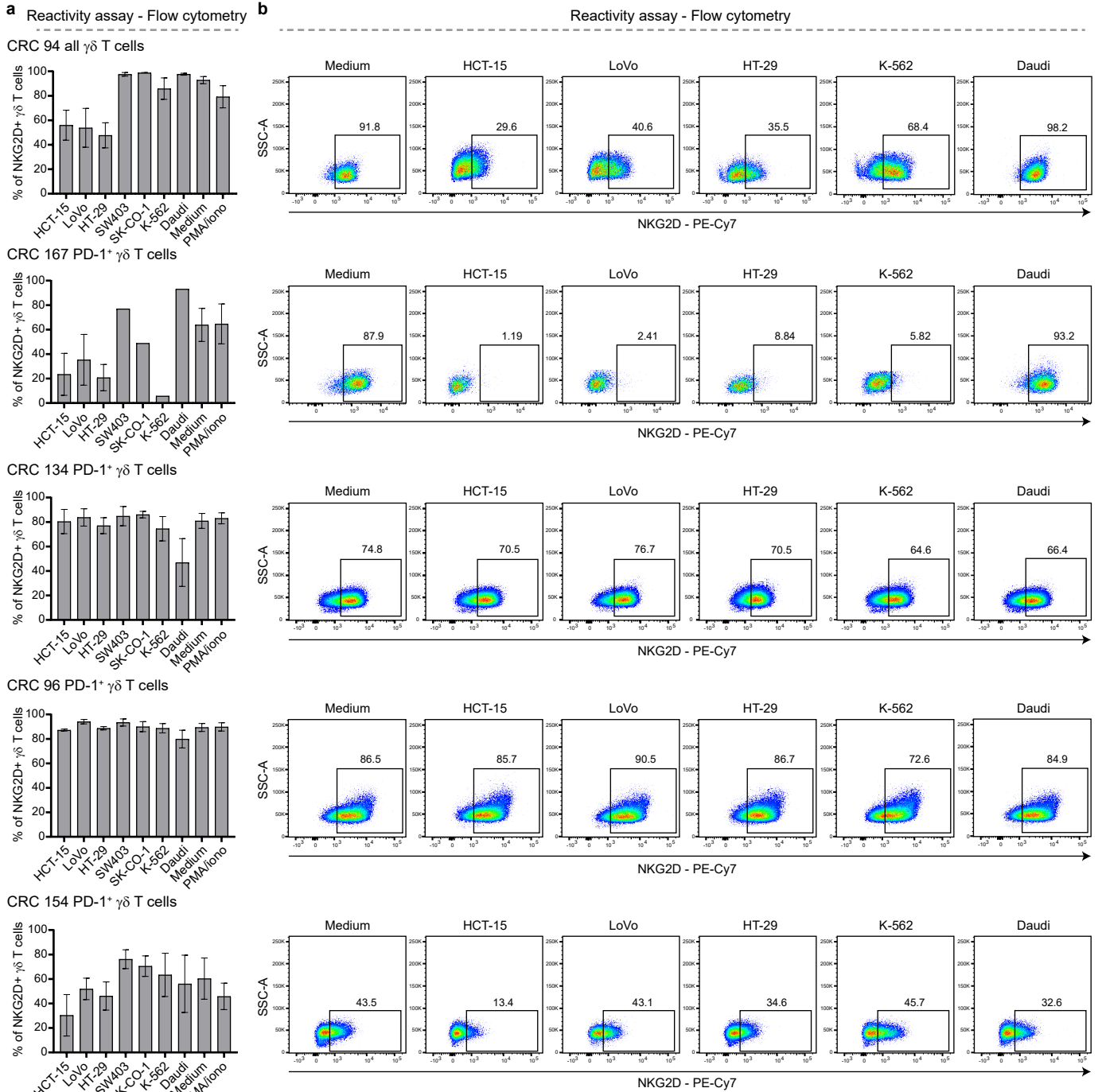
Extended Data Fig. 7



Extended Data Fig. 7. Tumor organoid characterization and reactivity assay readout.

- a.** Flow cytometry gating strategy on PDT0 cells for analysis of surface staining. Selected cells were gated on single, live cells before quantification of staining signal.
- b.** Histogram representation and count for surface staining of MHC-I, PD-L1, and β 2m expression on two PDT0 lines $B2M^{WT}$ and $B2M^{KO}$ after $IFN\gamma$ pre-stimulation. Staining with isotype antibodies for each fluorochrome (PE, APC and FITC) were included as negative control.
- c.** Flow cytometry gating strategy on $\gamma\delta$ T cell samples for analysis of intracellular staining to test antitumor reactivity upon PDT0 stimulation. Lymphocyte population was further gated on single cells, live and $CD3^+$ cells, $\gamma\delta$ TCR⁺ cells and $CD8^+$ as well as $CD8^-CD4^-$ cells. Reactivity of the sample was based on $IFN\gamma^+$ cells of the selected population.
- d.** Histogram representation and count for surface staining of NKG2D ligands MICA/B, ULBP1, ULBP2/5/6, ULBP3, and ULBP4 on two PDT0 lines $B2M^{WT}$ and $B2M^{KO}$ after $IFN\gamma$ pre-stimulation.
- e.** Flow cytometry gating strategy on $\gamma\delta$ T cell samples for analysis of intracellular staining after stimulation with PDT0s in the presence of NKG2D ligand blocking. Lymphocyte population was further gated on single cells, live and $CD3^+$ cells, followed by $\gamma\delta$ TCR⁺ and $CD8^+$ as well as $CD8^-$ cells. Reactivity of final population was based on $IFN\gamma^+$ or $CD107a^+$ cells.

Extended Data Fig. 8

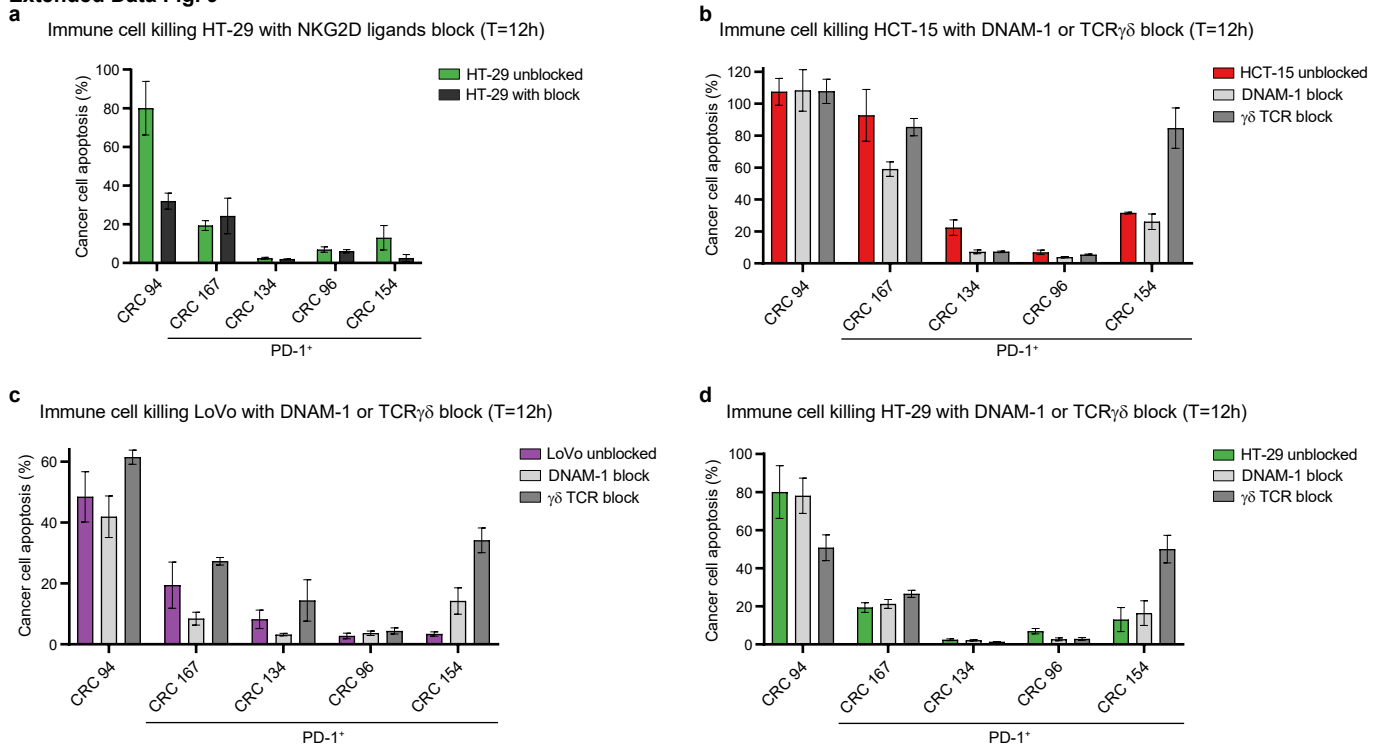


Extended Data Fig. 8. Surface expression of activating receptor NKG2D by PD-1⁺ $\gamma\delta$ T cells from MMR-d colon cancers upon co-culture with cancer cell lines.

a. Bar plots showing the expression of NKG2D on PD-1⁺ $\gamma\delta$ T cells from MMR-d colon cancers (n=5) after 18h co-culture of PD-1⁺ $\gamma\delta$ T cells with the cancer cell lines. Medium as negative control and PMA/ionomycin as positive control are included. Bars indicate mean \pm SEM. Data from four (CRC94), three (CRC167, CRC96), or two (CRC134, CRC154) independent experiments, depending on availability of $\gamma\delta$ T cells.

b. Flow cytometry plots showing the expression of NKG2D on PD-1⁺ $\gamma\delta$ T cells after 18h co-culture with HCT-15, LoVo, HT-29, K-562, and Daudi cells as compared to medium only. Gates indicate percentage of positive $\gamma\delta$ T cells.

Extended Data Fig. 9



Extended Data Fig. 9. Killing of cancer cell lines by PD-1⁺ $\gamma\delta$ T cells from MMR-d colon cancers in the presence of NKG2D ligand, DNAM-1, or $\gamma\delta$ TCR blocking.

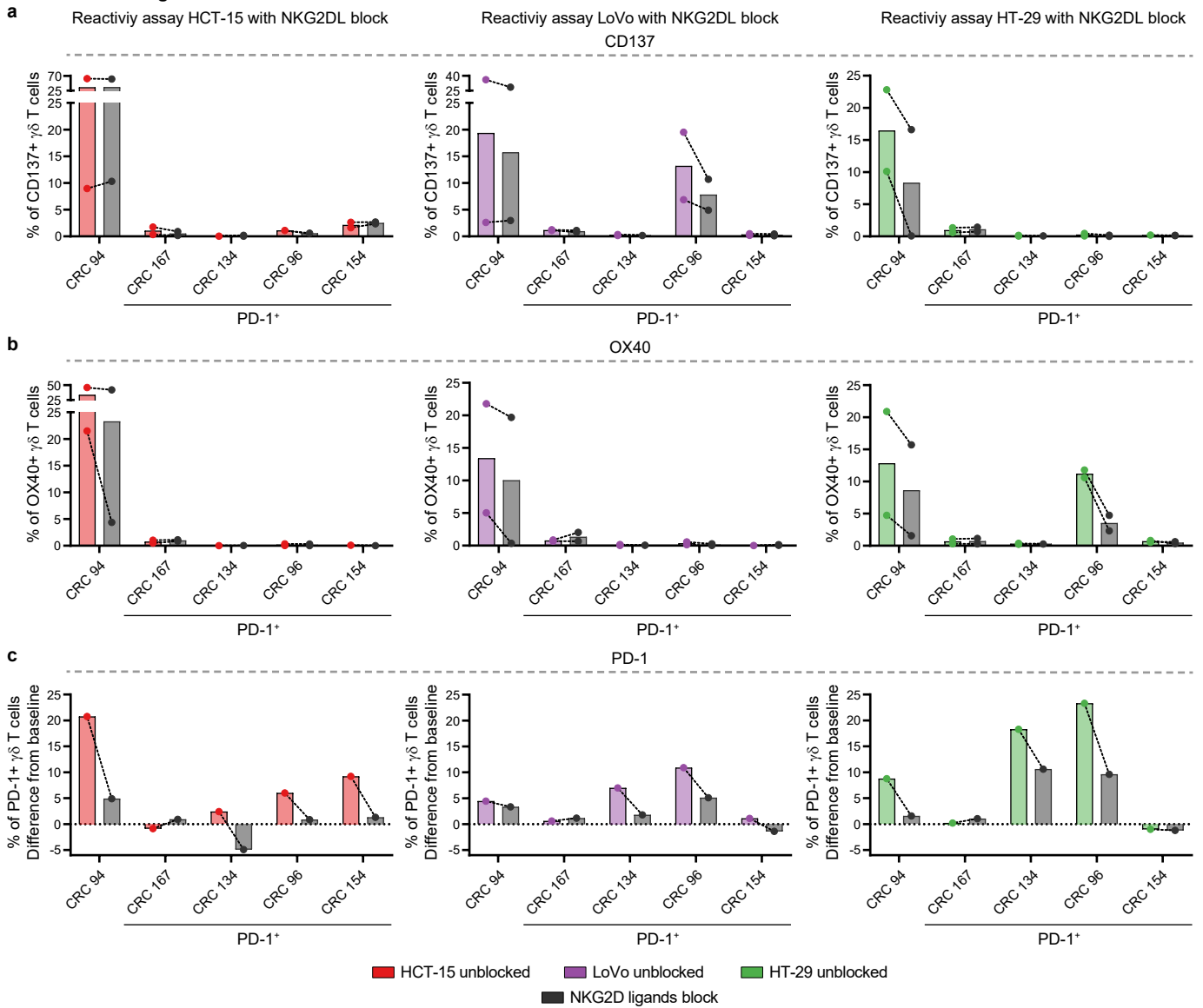
a. Bar plots showing the quantification of killing of HT-29 cells by $\gamma\delta$ T cells from MMR-d colon cancers (n=5) in the presence of blocking antibodies for NKG2D ligands as compared to the unblocked condition after 12h co-culture. Bars indicate mean \pm SEM of two wells with two images/well.

b. Bar plots showing the quantification of killing of HCT-15 cells by $\gamma\delta$ T cells from MMR-d colon cancers (n=5) in the presence of DNAM-1 or $\gamma\delta$ TCR blocking antibodies as compared to the unblocked condition after 12h co-culture. Bars indicate mean \pm SEM of two wells with two images/well.

c. As **(b)**, but for LoVo cells.

d. As **(b)**, but for HT-29 cells.

Extended Data Fig. 10



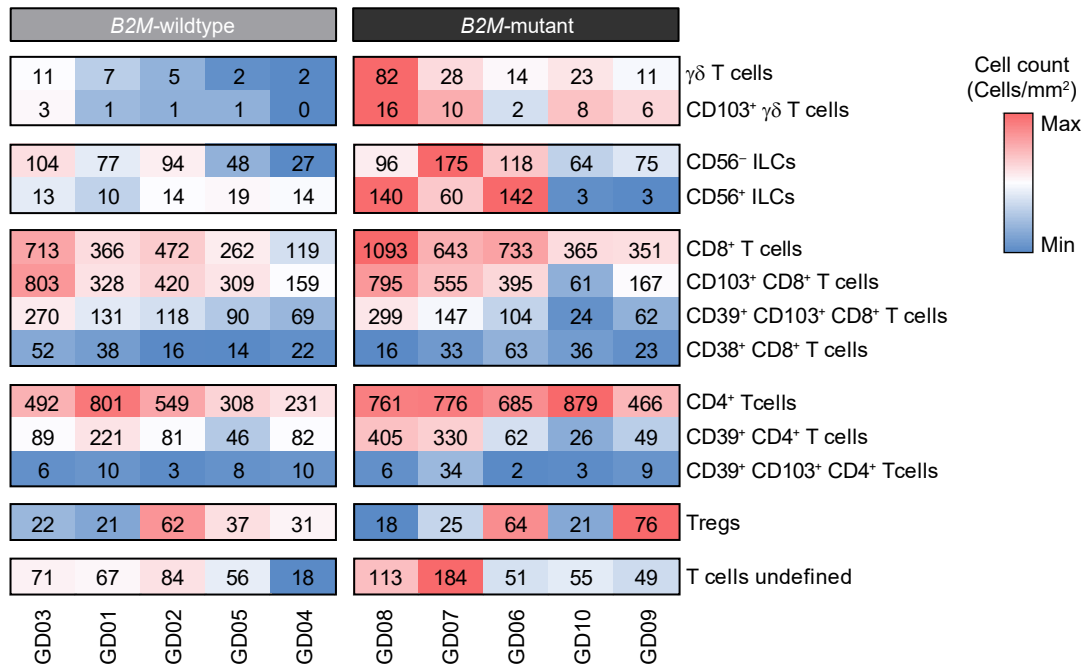
Extended Data Fig. 10. Reactivity towards cancer cell lines by PD-1⁺ $\gamma\delta$ T cells from MMR-d colon cancers in the presence of NKG2D ligand blocking antibodies.

a. Bar plots showing the percentage of CD137-positive $\gamma\delta$ T cells after 18h co-culture of PD-1⁺ $\gamma\delta$ T cells from MMR-d colon cancers (n=5) with HCT-15, LoVo, and HT-29 cells in the presence of blocking antibodies for NKG2D ligands. Lines indicate similar experiments. Data from two independent experiments.

b. Bar plots showing the percentage of OX40-positive $\gamma\delta$ T cells after 18h co-culture of PD-1⁺ $\gamma\delta$ T cells from MMR-d colon cancers (n=5) with HCT-15, LoVo, and HT-29 cells in the presence of blocking antibodies for NKG2D ligands. Lines indicate similar experiments. Data from two independent experiments.

c. Bar plots showing the expression of PD-1 on $\gamma\delta$ T cells as difference from baseline (medium) condition after 18h co-culture of PD-1⁺ $\gamma\delta$ T cells from MMR-d colon cancers (n=5) with HCT-15, LoVo, and HT-29 cells in the presence of blocking antibodies for NKG2D ligands. Lines indicate similar experiments.

Extended Data Fig. 11



Extended Data Fig. 11. Distribution of immune cell populations in *B2M*-wildtype and *B2M*-mutant colon cancers upon immune checkpoint blockade (ICB) by imaging mass cytometry.

Heatmap showing cell counts (cells/mm²) of different immune cell phenotypes from the imaging mass cytometric detection of *B2M*^{WT} (HLA class I-positive, n=5) and *B2M*^{MUT} (HLA class I-negative, n=5) MMR-d colon cancers upon ICB treatment. Hierarchical clustering was performed on the samples within the two groups. Color bar is scaled per major immune lineage.

Extended Data Table 1. Characteristics of clinical samples from 17 patients with MMR-deficient colon cancer.

Patient ID	Tumor location	MMR status	HLA class I status	β 2m status	Subgroup
CRC 1	Flexura lienalis	MMR-deficient	Positive	Positive	HLA+
CRC 2	Coecum	MMR-deficient	Defect	Mut pattern	HLA defect
CRC 3	Flexura hepatica	MMR-deficient	Positive	Positive	HLA+
CRC 6	Descendens	MMR-deficient	Defect	Defect	B2M defect
CRC 19	Ascendens	MMR-deficient	Defect	Defect	B2M defect
CRC 52	Ascendens	MMR-deficient	Defect	Mut pattern	HLA defect
CRC 67	Transversum	MMR-deficient	Positive	Positive	HLA+
CRC 84	Sigmoid	MMR-deficient	Defect	Defect	B2M defect
CRC 94 #	Sigmoid	MMR-deficient	Defect	Positive	HLA defect
CRC 96* #	Transversum	MMR-deficient	Defect	Defect	B2M defect
CRC 102	Ascendens	MMR-deficient	Defect	Positive	HLA defect
CRC 134* #	Ascendens	MMR-deficient	Defect	Defect	B2M defect
CRC 154* #	Flexura hepatica	MMR-deficient	Positive	Positive	HLA+
CRC 159*	Coecum	MMR-deficient	Defect	Mut pattern	HLA defect
CRC 167* #	Ascendens	MMR-deficient	Defect	Mut pattern	HLA defect
CRC 177	Flexura hepatica	MMR-deficient	Defect	Positive	HLA defect
CRC 195	Flexura hepatica	MMR-deficient	Defect	Positive	HLA defect

* Used for single-cell RNA-sequencing experiments, # Used for cell culturing experiments. B2M, β 2-microglobulin; MMR, mismatch repair.

Extended Data Table 2. Characteristics of patient-derived organoids from MMR-deficient colorectal cancer.

Sample	PDTO-1 wt	PDTO-1 <i>B2M</i> ko	PDTO-2 wt	PDTO-2 <i>B2M</i> ko
Tumor	CRC	CRC	CRC	CRC
MMR status	deficient	deficient	deficient	deficient
Biopsy / Resection	Resection	Resection	Biopsy	Biopsy
Biopsied lesion	Colon	Colon	Peritoneal	Peritoneal
Primary / Metastasis	Primary	Primary	Metastasis	Metastasis
Authenticated	Confirmed by SNP	Confirmed by STR	Confirmed by SNP	Confirmed by SNP
Mycoplasma	Negative	Negative	Negative	Negative
Characteristics	Luciferase transduced; parental line of PDTO-1 <i>B2M</i> ko	<i>B2M</i> knockout of PDTO-1 wt	Parental line of PDTO-2 <i>B2M</i> ko	<i>B2M</i> knockout of PDTO-2 wt
<i>B2M</i> knockout	-	sgRNA targeting <i>B2M</i> (GGCCGAGATGTCTCGCTCCG) cloned into LentiCRISPR v2 plasmid	-	sgRNA targeting <i>B2M</i> (GGCCGAGATGTCTCGCTCCG) cloned into LentiCRISPR v2 plasmid

Extended Data Table 3. Antibodies used for imaging mass cytometry of colon cancers.

Antibody	Metal	Clone	Supplier	Catalog number	Lot number	Incubation time (temperature)	Dilution
β-catenin	89Y	D10A8	CST	8480BF	8	Overnight (4°C)	1:100
CD103	168 Er	EPR4166(2)	Abcam	ab221210	GR3355784-7	5h (RT)	1:50
CD11b	144 Nd	D6X1N	CST	49420BF	4	5h (RT)	1:100
CD11c	176 Yb	EP1347Y	Abcam	ab216655	GR3357092-9	5h (RT)	1:100
CD14	163 Dy	D7A2T	CST	56082BF	2	5h (RT)	1:100
CD15	171 Yb	MC480	CST	4744BF	5	Overnight (4°C)	1:100
CD163	173 Yb	EPR14643-36	Abcam	93498BF	Not available	5h (RT)	1:50
CD20	142 Nd	E7B7T	CST	48750BF	9179056	Overnight (4°C)	1:100
CD204	164 Dy	J5HTR3	Thermo Fisher Scientific	14-9054-95	4338161	5h (RT)	1:50
CD3	153 Eu	EP449E	Abcam	ab271850	GR3341846-3	Overnight (4°C)	1:50
CD31	147 Sm	89C2	CST	3528BF	Not available	Overnight (4°C)	1:100
CD38	169 Tm	EPR4106	Abcam	ab226034	GR3378690-1	Overnight (4°C)	1:100
CD39	157 Gd	EPR20627	Abcam	ab236038	GR3274485-6	5h (RT)	1:100
CD4*	145 Nd	EPR6855	Abcam	ab181724	GR3285644-10	Overnight (RT)	1:100
CD45	149 Sm	D9M8I	CST	13917BF	11	Overnight (4°C)	1:50
CD45RO	165 Ho	UCHL1	CST	55618BF	2	Overnight (4°C)	1:100
CD56	167 Er	E7X9M	CST	99746BF	2	5h (RT)	1:100
CD57	151 Eu	HNK-1 / Leu-7	Abcam	ab269781	GR3373313-3	Overnight (4°C)	1:100
CD68	143 Nd	D4B9C	CST	76437BF	2	Overnight (4°C)	1:100
CD7	174 Yb	EPR4242	Abcam	ab230834	Not available	5h (RT)	1:100
CD8α	146 Nd	D8A8Y	CST	85336BF	Not available	5h (RT)	1:50
Cleaved caspase-3	172 Yb	5A1E	CST	9664BF	24	5h (RT)	1:100
D2-40	166 Er	D2-40	BioLegend	916606	B316467	Overnight (4°C)	1:100
FOXP3	159 Tb	D608R	CST	12653BF	8	Overnight (4°C)	1:50
Granzyme B	150 Nd	D6E9W	CST	46890BF	3	5h (RT)	1:100
Histone H3	209	D1H2	CST	4499BF	Not available	Overnight (4°C)	1:50
HLA-DR	141 Pr	TAL 1B5	Abcam	ab176408	GR3384096-1	5h (RT)	1:100
ICOS	161 Dy	D1K2T™	CST	89601BF	4	5h (RT)	1:50
IDO	162 Dy	D5J4E™	CST	86630BF	7	Overnight (4°C)	1:100
Ki-67	152 Sm	8D5	CST	9449BF	11	Overnight (4°C)	1:100

LAG-3	155 Gd	D2G40™	CST	15372BF	Not available	5h (RT)	1:50
p16ink4a	175 Lu	D3W8G	CST	92803BF	2	Overnight (4°C)	1:100
Pan-keratin	198 Pt	C11 and AE1/AE3	CST / BioLegend	4545BF / 914204	12 / B302316	Overnight (4°C)	1:50
PD-1	160 Gd	D4W2J	CST	86163BF	7	5h (RT)	1:50
PD-L1	156 Gd	E1L3N ^R	CST	13684BF	17	Overnight (4°C)	1:50
T-bet	170 Er	4B10	BioLegend	644825	B298378	5h (RT)	1:50
TCR δ^*	148 Nd	H41	Santa Cruz	sc-100289	D3021	Overnight (RT)	1:50
TGF β	115 In	TB21	Thermo Fisher Scientific	MA5- 16949	151471	5h (RT)	1:100
TIM-3	154 Sm	D5D5R™	CST	45208BF	9	5h (RT)	1:100
Vimentin	194 Pt	D21H3	CST	5741BF	9	Overnight (4°C)	1:50
VISTA	158 Gd	D1L2G™	CST	64953BF	7	5h (RT)	1:100

*Detection with metal-conjugated secondary antibodies. CST, Cell Signaling Technology.

Extended Data Table 4. Antibodies used for immunophenotyping of $\gamma\delta$ T cells by flow cytometry.

Antibody	Fluorochrome	Clone	Supplier	Catalog number	Lot number	Dilution
CD16	PE	B73.1	BD Biosciences	332779	9045985	1:60
CD103	FITC	Ber-ACT8	BD Biosciences	550259	2332847	1:10
CD122/IL-2R β	BV421	TU27	BioLegend	339010	B313155	1:20
CD132/IL-2R γ	APC	TUGh4	BioLegend	338608	B293032	1:80
CD161	BV605	DX12	BD Biosciences	563863	7030586	1:20
CD25/IL-2R α	PE-Cy7	M-A251	BD Biosciences	557741	9301660	1:25
CD215/IL-15R α	PE	JM7A4	BioLegend	330208	B265801	1:80
CD226/DNAM-1	BV510	DX11	BD Biosciences	742494	9203072	1:150
CD3	Am Cyan	SK7	BD Biosciences	339186	9161745	1:20
CD32	APC	FLI8.26	BD Biosciences	559769	184743	1:20
CD38	PE-Cy7	HIT2	eBioscience	25-0389-42	4319912	1:200
CD39	APC	A1	BioLegend	328210	B249211	1:60
CD45RA	FITC	L48	BD Biosciences	335039	8227525	1:30
CD45RA	PE-Dazzle594	HI100	Sony	2120730	126470	1:20
CD45RO	PerCP-Cy5.5	UCHL1	Sony	2121110	138351	1:20
CD56	APC-R700	NCAM16.2	BD Biosciences	565139	5251693	1:150
CD64	FITC	10.1	BD Biosciences	555527	58058	1:20
CD69	PerCP-Cy5.5	FN50	BioLegend	310925	B266970	1:200
CD8 α	BV605	SK1	BD Biosciences	564115	7092	1:100
CD94	BV605	HP-3D9	BD Biosciences	743950	7138571	1:200
GITR	PE	108-17	BioLegend	371204	B244963	1:50
Granzyme B*	PE	GB11	eBiosciences	12-8899-41	1928380	1:50
KIR2DL1	PE	HP3-E4	BD Biosciences	556063	86798	1:20
KIR2DL1/S1	PE	EB6	Beckman Coulter	A09778	12	1:50
KIR2DL2/L3/S2	PE	GL183	Beckman Coulter	IM2278U	200051	1:50
KIR2DL4	PE	181703	R&D Systems	FAB2238P	AAHO0209081	1:10
KIR2DS4	PE	FES172	Beckman Coulter	IM3337	200037	1:40
KIR3DL1	PE	DX9	BD Biosciences	555967	121769	1:80
KIR3DL1/S1	PE	Z27	Beckman Coulter	IM3292	200044	1:20
KIR3DL2	PE	#539304	R&D Systems	FAB2878P	ADBO0217051	1:10
LAG3	PE-Cy7	11C3C65	BioLegend	369309	B289009	1:100
NKG2A	APC	z199	Beckman Coulter	A60797	200046	1:30
NKG2C	PE	134591	Beckman Coulter	FAB138P	LCN0818011	1:20
NKG2D	PE-Cy7	1D11	BD Biosciences	562365	9045733	1:300

NKp44	APC	P44-8	BioLegend	325109	B160899	1:20
NKp46	PE	9E2	BioLegend	331907	B150121	1:20
PD-1	PE	MIH4	eBioscience	12-9969-42	1952441	1:30
Perforin*	PE-Cy7	dG9	BioLegend	308125	B215704	1:20
TCR $\gamma\delta$	BV421	11F2	BD Biosciences	744870	9340519	1:80
TCR $\gamma\delta$	BV650	11F2	BD Biosciences	745359	7222894	1:40
TCR V δ 1	FITC	TS8.2	Invitrogen	TCR2730	UH286015	1:50
TCR V δ 2	PerCP-Cy5.5	B6	BioLegend	331424	B279957	1:200
TIGIT	APC	1D11	BD Biosciences	562365	9045733	1:300
Live/dead	nIR	n.a.	Life Technologies	L10119	1808830	1:1000

*Detected intracellularly