Title

Modelling cancer immunomodulation using epithelial organoid cultures

Authors

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Introduction

Colorectal cancer (CRC) is among the most common cancers worldwide[1]. While early CRC stages are highly treatable by surgical removal, later stages are usually incurable[2]. CRC arises through a multi-step process from small lesions of the epithelium of the large intestine. These lesions grow into adenomas with low grade dysplasia that progress into high grade dysplasia, eventually giving rise to infiltrating carcinomas[3]. Genetic mutations in signalling pathways such as the canonical Wnt signalling are the molecular basis of CRC[4]. However, the interaction of the tumour with its microenvironment is another critical hallmark[5]. Cancer cells remodel their microenvironment (e.g. fibroblasts, the vasculature and immune cells) to support tumour growth[6]. Infiltrating immune cells (ICs) such as cytotoxic T cells (CTLs) or macrophages play a crucial role by generating different

32 immune responses such as anti-tumour cytotoxicity (the former) or tumour-promoting chronic
33 inflammation (the latter)[7]. As such, escape from the surveilling immune system has been
34 recognised as one of the hallmarks of cancer[5]. Cancer cells undergo a process called
35 immunoediting and silence anti-tumour responses, for example, by preventing T-cell activation
36 through stimulation of inhibitory cell surface receptors such as CTL-associated antigen (CTLA)-
37 4 or programmed death (PD)1[6,8]. Overcoming this active immunomodulation by tumour cells has
38 become a major therapeutic target[9]. However, tumour heterogeneity, such as differential CTL
39 infiltration, could influence therapeutic efficiency of anti-tumour drugs by mediating drug
40 resistance[6]. Developing *ex vivo* model systems to characterise the communication of the tumour
41 with its environment is therefore of great importance. Organoid cultures grown from different
42 epithelial tissues serve as an excellent tool to study tissue homeostasis and disease[10]. Furthermore,
43 organoid biobanks of multiple epithelial organ systems have been established and tumor-derived
44 organoids have successfully been used as platforms for screenings of different drugs to predict
45 patient response[11]. Here we describe the establishment of a method to model antigen-specific
46 epithelial-cell killing and cancer immunomodulation and *in vitro* using CRC organoids co-cultured
47 with CTLs.
48
49 **Results**
50 ***CRC organoids express immunomodulatory molecules***
51 We first assessed whether CRC organoids expressed immunomodulatory molecules in established
52 long-term expanded cultures. Therefore, we compared gene expression of T-cell-specific
53 immunomodulators in CRC organoids to the expression levels found in normal colon organoids
54 using a transcriptome dataset generated using our 'living organoid biobank' of CRC patients[12]. On
55 average, transcription of genes associated with T-cell stimulation such as *TNFSF4* or *TNFSF9* was
56 not altered in CRC organoids compared to normal colon organoids (Fig. 1a). However, expression
57 of human leukocyte antigen (HLA) genes *HLA-A* and *HLA-C*, encoding major histocompatibility
58 complex class (MHC)-I molecules that present antigens to T cells, were significantly
59 downregulated in CRC organoids (Fig. 1a), a well-described phenomenon found in cancers[13].
60 Expression of genes associated with inhibition of T-cell function was either significantly
61 upregulated such as *BTLA*, significantly downregulated such as *CD80*, *CD86* or LGALS9 or not
62 altered at all such as *CD274* (encoding PD-L1), *PDCD1LG2* (encoding PD-L2) (Fig. 1a). When

assessing expression levels of immunomodulatory molecules on individual organoids, CRC organoids largely clustered together showing heterogeneous down regulation of *HLA-A*, *HLA-C* and *LGALS9* compared to healthy colon organoids (Fig. 1b). However, expression of immunoinhibitory genes *CD274*and *PDCD1LG2*, for instance, was highly upregulated in some CRC organoids in comparison to the matched normal colon organoid cultures, reflecting previously reported preservation of tumour heterogeneity in organoids[12] (Fig. 1b). These molecular signatures provide a basis for further investigation of tumour immunogenicity and its association with other characteristics of the tumour.

Four of the most commonly mutated genes in CRC are *APC*, *P53*, *KRAS* and *SMAD4*, reflecting the stepwise progression of the normal intestinal epithelium into a metastatic carcinoma[14]. Introduction of these cancer mutations into human intestinal organoid cultures using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 demonstrated that this process can be mimicked *in vitro* and upon xenotransplantation into mice[15,16]. Using colon organoids carrying one or more of these cancer mutations, we investigated whether up-regulation of PD-L1 was associated with a certain mutational status. Additionally, we exposed mutant organoids and their wild-type control organoid line to Interferon (IFN)-γ, which is secreted by T cells and can trigger increased expression of immunomodulatory molecules such as PD-L1[17]. Subsequently, we assessed PD-L1 expression by quantitative polymerase chain reaction (qPCR) and flow cytometry (Fig. 1 c,d). In the absence of IFN-γ, organoids carrying triple ($APC^{KO/KO}$, $P53^{KO/KO}$, $KRAS^{G12D/+}$) and quadruple mutations ($APC^{KO/KO}$, $P53^{KO/KO}$, $KRAS^{G12D/+}$ and $SMAD4^{KO/KO}$) showed lower *CD274* gene expression in comparison to control wild-type organoids (Fig. 1 c). Overall, PD-L1 expression was low in untreated organoid lines (Fig. 1 c,d). However, PD-L1 expression was dramatically upregulated in IFN-γ-treated organoids both on transcript and protein level (Fig. 1 c,d). These data demonstrate that CRC organoids express immunomodulators and that this expression is regulated in a similar way as previously shown for tissue *in vivo.*

CRC organoids as tools for assessment of antigen specific killing by CD8$^+$ T cells

We next aimed at establishing a co-culture system for CRC organoids and CTLs to model antigen-specific killing of tumour cells *in vitro*. For this, we used αβ T cells carrying a transgenic T-cell receptor (TCR) recognizing an HLA-A2-restricted Wilms tumour (WT)1-derived peptide[18,19]. Therefore, we first screened CRC organoids from the 'living biobank'[12] as well as newly generated

94 CRC organoids for HLA-A2 expression using flow cytometry. Interestingly, we found three CRC
95 organoid lines that showed partial downregulation of HLA-A2 (Supplementary Fig. 1a). We were
96 able to purify HLA-A2$^+$ and HLA-A2$^-$ CRC organoids and successfully established cultures from
97 both populations (Fig. 2b). We confirmed stable MHC-I downregulation in HLA-A2$^-$ CRC
98 organoids, as IFN-γ stimulation did not trigger HLA-A2 re-expression (Supplementary Fig. 1b).
99 Next, we pulsed these CRC organoid lines with WT1 peptide and, subsequently, co-cultured them
100 for 48 hours with peptide-specific T cells. Following co-culture, we found that HLA-A2$^-$ CRC
101 organoids did survive irrespective of whether pulsed with the peptide or not (Fig. 2c). However,
102 only the HLA-A2$^+$ CRC organoids without prior peptide incubation survived co-culture (Fig. 2c).
103 Peptide-pulsed HLA-A2$^+$ CRC organoids were effectively killed by the peptide-specific T cells
104 providing a proof-of-principle that organoids can be utilised to study anti-tumour response by
105 cytotoxic T cells *in vitro*.
106 To further confirm antigen-specificity in our 'killing' assay system, we improved our co-
107 culture method by transfecting HLA-A2$^+$ CRC organoids with a construct expressing
108 mNeonGreen-tagged histone H2B and staining T cells with CellTracker violet to allow for long-
109 term tracking of both cell types (Methods). We then pulsed HLA-A2$^+$ CRC organoids with either
110 the WT1 peptide or with an EBV-derived peptide (Methods) and co-cultured the organoids with T
111 cells carrying either a WT1- or an EBV-specific TCR. Here, only organoids pulsed with the
112 cognate peptide were efficiently killed by the T cells (Fig. 2d, Supplementary Movies 1 and 2).
113 Testing for IFN-γ production by the T cells in the co-culture using enzyme-linked immunosorbent
114 assay (ELISA) confirmed antigen-specific organoid killing by the T cells (Fig. 2e). In order to
115 better follow the kinetics of the organoid killing, we applied to a fluorescent dye (NucRed Dead
116 647, Methods), which specifically stains apoptotic cells, and performed live confocal imaging on
117 the co-culture (Fig. 2f). We then quantified organoid killing by assessing co-localization of
118 NucRed Dead dye with H2B-mNeonGreen (Methods). Significant co-localisation of both labels
119 and, hence, organoid killing, was only observed when peptide-pulsed HLA-A2$^+$ CRC organoids
120 were co-culture with the respective peptide-specific T cells (Fig. 2g). Furthermore, T cells
121 infiltrating into the epithelium of the organoids could be readily detected in this co-culture
122 condition (Fig. 2h). In conclusion, T cells efficiently killed co-cultured CRC organoids in an
123 antigen-specific manner.
124

125 **Discussion**

126 Here we demonstrate that epithelial organoids can be used faithfully to recapitulate the interaction
127 between tumour tissue and the immune system. Also, using our co-culture assay, we set a first step
128 in rebuilding the tumour microenvironment *in vitro*. Further addition of other components of this
129 microenvironment (such as fibroblasts, natural killer cells, myeloid-derived suppressor cells, B
130 cells) may shed light on the complex interactions between the different cell types leading to
131 immune evasion of the tumour. Lastly, this co-culture system can be used as a scaffold for T-cell
132 expansion for cancer immunotherapy or for drug-screens testing applicability of certain
133 immunotherapies, for instance, chimeric antigen receptor (CAR)- or TCR transgenic T cells,
134 antibody-dependent cell-mediated cytotoxicity (ADCC) or antibody-dependent cellular
135 phagocytosis (ADCP) inducing antibodies directed at the tumour, to different tumours and
136 different patients.

137

138 **Methods**

139 ***Procurement of human material and informed consent***

140 Colonic tissues (both normal colon and tumour tissue) were obtained from the Departments of
141 Surgery and Pathology of the Diakonessenhuis hospital, Utrecht, the Netherlands. All patients
142 included in this study were diagnosed with CRC. Informed consent was signed by all included
143 patients. Collection of tissue was approved by the medical ethical committee (METC) of the
144 Diakonessenhuis hospital, in agreement with the declaration of Helsinki and according to Dutch
145 and European Union legislation.

146

147 ***Organoid generation and cultures***

148 Epithelial organoid lines were derived from healthy colon or tumor tissue as previously
149 described[12,20]. In brief, healthy colonic crypts were isolated by digestion of the colonic mucosa in
150 chelation solution (5.6 mM Na_2HPO_4, 8.0 mM KH_2PO_4, 96.2 mM NaCl, 1.6 mM KCl, 43.4 mM
151 Sucrose, and 54.9 mM *D*-Sorbitol, Sigma) supplemented with dithiotreitol (0.5 mM, Sigma) and
152 EDTA (2 mM, in-house), for 30 minutes at 4°C. Colon crypts were subsequently plated in
153 basement membrane extract (BME; Cultrex PC BME RGF type 2, Amsbio) and organoids were
154 grown in human intestinal stem cell medium (HISC), which is composed of Advanced Dulbecco's
155 modified Eagle medium/F12 supplemented with penicillin/streptomycin, 10 mM HEPES and

156 Glutamax (all Gibco, Thermo Fisher Scientific) with 50% Wnt3a conditioned medium (in-house),
157 20% R-Spondin1 conditioned medium (in-house), 10% Noggin conditioned medium (in-house), 1
158 x B27, 1,25 mM n-acetyl cysteine, 10 mM nicotinamide, 50 ng/mL human EGF, 10 nM Gastrin,
159 500 nM A83-01, 3 μM SB202190, 10 nM prostaglandine E2 and 100 μg/mL Primocin (Invivogen).
160 Tumor specimens were digested to single cells in collagenase II (1 mg/mL, Gibco, Thermo
161 Scientific), supplemented with hyaluronidase (10 μg/mL) and LY27632 (10 μM) for 30 minutes
162 at 37°C while shaking. Single tumor cells were plated in BME and organoids were cultured in
163 HICS minus Wnt conditioned medium and supplemented with 10 μM LY27632 at 37°C.
164
165 ***Organoid transfection***
166 CRC organoids were dissociated into small clumps using TrypLE and then transduced with H2B-
167 mNeonGreen (pLV-H2B-mNeonGreen-ires-Puro), as previously described[21].
168
169 ***T cells***
170 Generation of αβ T cells carrying a transgenic TCR recognizing an HLA-A2-restricted WT1-
171 derived peptide were described elsewhere[18]. Briefly, TCRα and β chains were cloned from raised
172 tetramer positive T cell clones. Subsequently, $CD8^+$ αβ TCR T cells were transduced using
173 retroviral supernatant from Phoenix-Ampho packaging cells that were transfected with gag-pol,
174 env, and pBullet retroviral constructs containing the cloned TCR genes.
175
176 ***Organoid-T cell co-culture and live cell imaging***
177 Organoids stably transfected with H2B-mNeonGreen were split and digested a 5 to 7 days prior to
178 co-culture and seeded at a density of 5000 cells per 10 μL of BME (25,000 cells per well in a 12-
179 well cell culture plate). Two days prior to co-culture, T cells were starved from IL-2. One day prior
180 to co-culture, organoids were stimulated with IFN-γ at indicated concentrations.
181 Organoids were pulsed with TCR-specific peptide (ProImmune) for 2 hours at 37°C prior to co-
182 culture. Organoids and T cells were harvested and taken up in T cell medium, supplemented with
183 10% BME, 100 IU/mL IL-2 and NucRed Dead 647 (Thermo Fischer). Where indicated, anti-PD1
184 blocking antibodies (2 μg/mL) were added to the co-culture. Cells were plated in glass-bottom 96-
185 well plates and co-cultures were imaged using an SP8X confocal microscope (Leica).
186

187 ***Flow cytometry***

188 APC-labelled pentamers to the EBV-derived, HLA-2:02 restricted peptide FLYALALLL
189 (ProImmune) where used to sort pentamer$^+$ CD8$^+$ CD3$^+$ T cells from PBMCs isolated from
190 buffycoats from healthy individuals. Cells were sorted as single cells into 96-well plates using a
191 BD FACS Aria (BD Biosciences) cytometer. For flow cytometry, the following antibodies were
192 used (all anti-human): CD8–PE (clone RPA-T8), CD45–PerCP-Cy5.5 (2D1), CD274 (PD-L1)–
193 APC (MIH1) (all BD Biosciences), CD279 (PD-1)–PE (EH12.2H7, Biolegend), HLA-A2–PE
194 (BB7.2, Santa Cruz).

195

196 ***Quantitative polymerase chain reaction (qPCR)***

197 For qPCR analysis, RNA was isolated from organoids using the RNAeasy kit (QIAGEN)
198 according to the manufacturer's protocol. PCR analysis was performed using the SYBR Green
199 Reagent (Biorad). PCR reactions were performed in duplicate with a standard curve for every
200 primer. Primers were designed using the NCBI primer design tool. Primers used in this study:
201 *GAPDH* forward (GTC GGA GTC AAC GGA TT), *GAPDH* reverse (AAG CTT CCC GTT CTC
202 AG), *HPRT* forward (GGC GTC GTG ATT AGT GAT), *HPRT* reverse (AGG GCT ACA ATG
203 TGA TGG), *CD274* forward (TGC AGG GCA TTC CAG AAA GAT), *CD274* reverse (CCG
204 TGA CAG TAA ATG CGT TCAG).

205

206 ***Transcriptional profiling***

207 Microarray analysis of biobank organoids was performed as described elsewhere[12].

208

209 ***Enzyme linked immunosorbent assays (ELISA)***

210 Culture supernatants were kept at –20°C and ELISA was performed for indicated cytokines using
211 ELISA MA Standard (Biolegend) according to manufacturer's protocol.

212

213 ***Image analysis***

214 Image analysis was done using Imaris software package (Bitplane). In brief, threshold for positive
215 staining was set on negative controls. A co-localization channel was made for H2B-neon and
216 NucRed Dead 647 signals. Cell death was quantified as percentage of H2B-mNeonGreen$^+$ voxels
217 co-localising with NucRed Dead signal.

218

219 ***Bioinformatics analysis***

220 Bioinformatics analysis of gene-expression data from microarray experiments[12] was performed
221 using standard packages (*i.e.* gplots) in R version 3.4.0 (R Foundation, https://www.r-project.org)
222 and RStudio version 1.0.143 (https://www.rstudio.com).

223

224 ***Statistical analysis***

225 All experiments were repeated at least three times unless otherwise indicated. All data were shown
226 as mean ± SEM. Statistical significance was analyzed by either ANOVA or two-tailed Student's
227 *t*-test using either Graphpad Prism 6 or Microsoft Excel 2010.

228

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281

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292

293 **Author contributions**

294 Y.B.E. and K.K. designed, performed and analysed the experiments and wrote the manuscript.
295 Y.B.E. performed image analysis. K.K. performed bioinformatics analysis. P.A., E.d.J. and K.E.B.
296 assisted with experiments. J.D. generated cancer gene-mutant organoid lines. J.v.G. isolated
297 tumour and normal tissue from resected material. A.P. and N.S. performed surgery. I.J.G., Z.S.
298 and J.K. provided WT1 peptide and WT1 peptide-specific transgenic TCR αβ T cells. R.G.J.V.
299 organised tissue collection. K.K. and H.C. acquired funding. H.C. supervised the project and wrote
300 the manuscript. All of the authors commented on the manuscript.
301

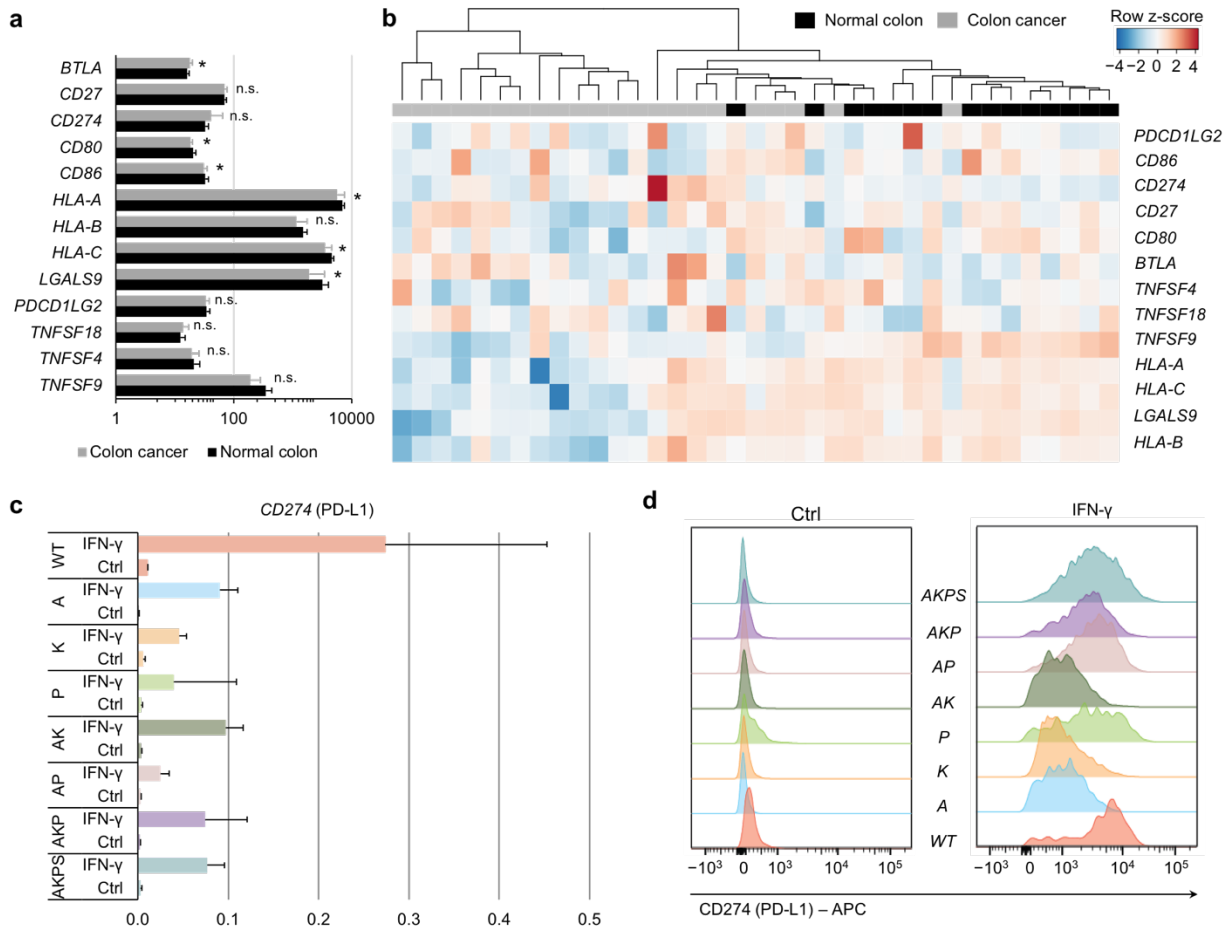

Fig. 1 | CRC organoids express immunomodulatory molecules. a,**b**, Normal colon and CRC organoid lines were generated in a patient-specific manner and RNA was extracted and analysed using Affymetrix single transcript microarrays. Average gene expression of different immunomodulators in normal colon and CRC organoid lines; n.s., non-significant; *, $p < 0.05$ (**a**). Hierarchical clustering of the individual normal colon and CRC organoid lines in the 'living biobank' displaying gene expression of selected immunomodulators. Color gradients represent z valued of each row (gene transcripts). **c-d**, Human colon organoid lines genetically engineered to carry one or more mutations found in CRCs. Expression levels of CD274 (PD-L1) in organoid lines (n = 2) at steady state (Ctrl) and upon stimulation with 20 ng/mL recombinant human IFN-γ assessed by quantitative PCR (**c**) and flow cytometry (**d**). A, *APC*$^{KO/KO}$; N.D., not detected; K, *KRAS*$^{G12D/+}$: P, *P53*$^{KO/KO}$; S, *SMAD4*$^{KO/KO}$, WT, wild-type.

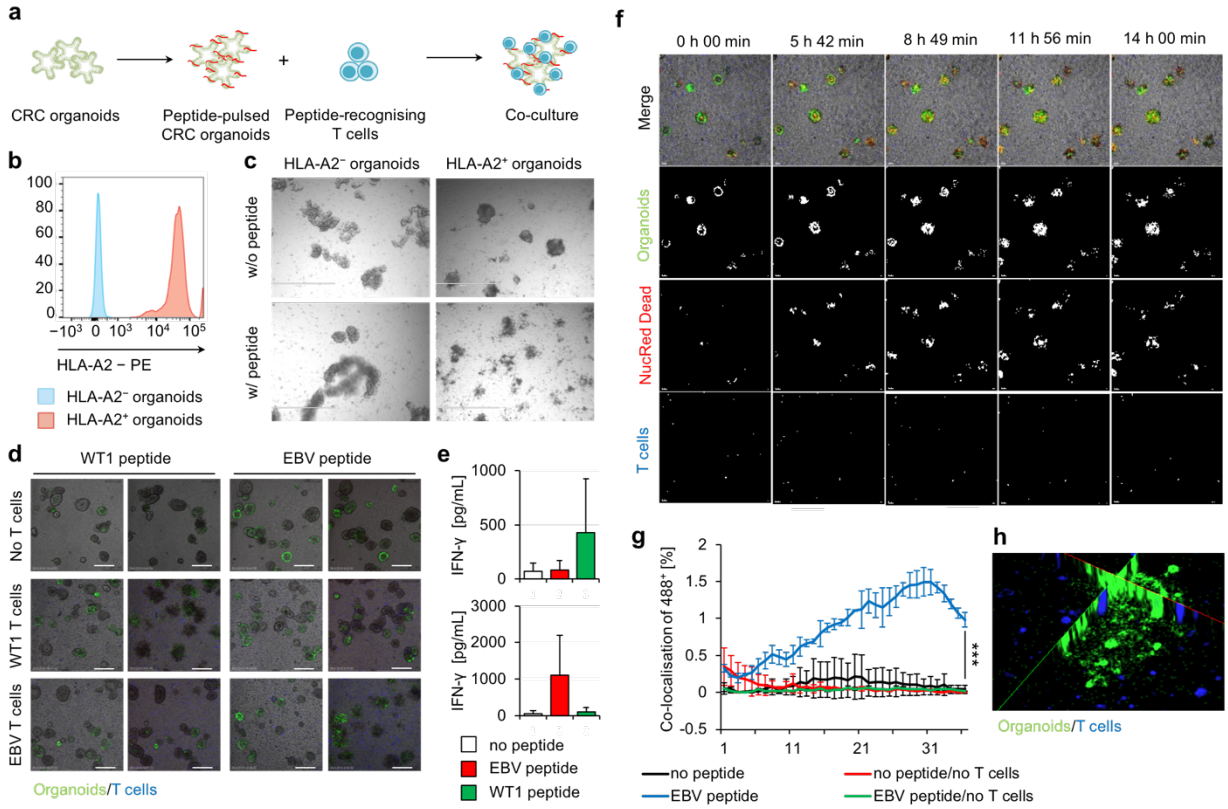

Fig. 2 | CRC organoids as tools for assessment of antigen specific killing by CD8⁺ T cells. a, Experimental scheme. **b**, Flow cytometry analysis of HLA-A2 expression in cloned HLA-A2⁺ and HLA-A2⁻ lines. **c**, Brightfield images of HLA-A2⁺ and HLA-A2⁻ CRC organoids co-cultured with transgenic T cells expressing the WT1 peptide-specific T-cell receptor for 48 hours; scale bars: 1 mm. **d,** Images showing HLA-A2⁺ CRC organoids transfected with H2B-neon and pulsed with indicated peptides at the beginning of co-culture (left panels) and after 18-hour co-culture with indicated peptide-specific T cells (right panels); scale bars: 70 µm. **e**, IFN-γ production by WT1 (top) and EBV (bottom) peptide-specific T cells as measured by ELISA of supernatants collected after 18-hour co-culture with HLA-A2⁺ CRC organoids pulsed with indicated peptides. **f**, Live-cell imaging stills of a co-culture experiment with H2B-mNeonGreen-transfected HLA-A2⁺ CRC organoids pulsed with EBV peptide and co-cultured for 14 hours with an EBV-specific T-cell clone. Images were taken at the indicated time points. **g**, Quantification of CRC organoid killing by specific T cells as assessed by co-localization of H2B-mNeonGreen and NucRed Dead dye. Graphs are representative of multiple repeated experiments with either EBV peptide and EBV T-cell- or WT1 peptide and WT1 T-cell co-cultures. **h**, Representative projection image of T cells

333 (blue) infiltrating a peptide-pulsed CRC organoid as recorded during the live-cell imaging
334 experiments.