SUPPLEMENTAL FIGURES

Suppl. Figure 1. Schematic representation of Cet-ZA ADC synthesis. 1. ZA and EDC generate a phosphodiester (acylurea) intermediate; 2. This acylurea reacts with imidazole generating a reactive phosphorimidazolide that (3.) reacts with the free amino-groups of cetuximab (Erbitux®); 4. this reaction generates an intermediate highly reactive that dissociates into free imidazole and a phosphoramidate composed of ZA conjugated with cetuximab (Erbitux®).

Suppl. Figure 2. Cet and Cet-ZA ADC internalization in CRC. A.The HCT116 cell line was incubated at RT for 1h with Cet (left) or Cet-ZA (right) followed by APC-α-hIg antiserum (red, upper images) and Syto16 (blue, central images), merged in the lower images. Bar: 25µm. Other samples were incubated for 24h at 37°C (B) with Cet (upper images) or Cet-ZA (lower images) as indicated, permeabilized and stained with APC-α-hIg antiserum (red), Syto16 (blue) and anti-LAMP-1 mAb followed by AlexaFluor535-conjugated GAM (green); merged images in the upper right and lower right pictures. Images were taken with the FV500 confocal Laser Scanning Microscope (40X magnification), in sequence mode and data were analyzed with FluoView 4.3b computer software. Picture are shown in pseudocolor as red/green fluorescence vs nuclei in blue. In (B) enlargements of the squares in the upper merged pictures are shown. Yellow dots (white arrows) indicate colocalization of LAMP-1 with either Cet or Cet-ZA.

Suppl. Figure 3. Histochemical (IHC) characterization of CRC organoids. Four µm thick section from Histochoice fixed and paraffin-embedded CRC organoids (n=9, represented out of 13 tested) stained with the mouse anti-MUC2 (1:400), rabbit anti-VIL1 (1:400), or the mouse anti-CGHA (1:1000) antibodies, developed with Leica Mixed DAB Refine reagent, and counterstained with Hematoxylin. Digital images captured under 20X or 40X objective magnification (Leica Aperio AT2 scanner) and analyzed with the Leica Aperio Scan Scope software.

Suppl. Figure 4. Quantification of BTN3A1, BTN2A1 and Vδ2 expression in CRC tissues and organoids. A. Upper pictures: IHC of BTN3A1 or BTN2A1 expression in a representative CRC. Lower pictures: representative image analysis of BTN3A1 or BTN2A1 cytoplasmic expression quantified by the Cytoplasmic V2 macro derived from the algorithm of Image Scope 12.3 software. Images show the classification of cellular cytoplasmic staining in pseudocolors and the intensity is denoted by a variation from brown to yellow. B. Upper pictures: Representative IHC of BTN3A1 or BTN2A1 expression in organoids. Lower pictures: representative image analysis as in A. C. Upper

Benelli R, *et al. J Immunother Cancer* 2022; 10:e005660. doi: 10.1136/jitc-2022-005660

picture: Vδ2 expression by IHC in a representative CRC. Lower picture: the Nuclear V9 macro from the algorithm of Image Scope 12.3 software (Leica) was applied to refer the number of Vδ2 T cells to total cell count.

Suppl. Figure 5. Cet-ZA ADC-elicited cytotoxicity on CRC organoids evaluated by fluorescence and imaging. A. Upper panels: co-cultures of CRC organoid (18-025) and autologous Vδ2 T cells, with 100nM of C. LIVE Tox Green fluorescent probe, either without (CTR) or with stimuli (2µg/ml Cet, 5µM ZA, 2µg/ml Cet-ZA), as indicated. Lower panels: parallel samples with anti(α)-Vδ2 and anti(α)-CD16 mAbs (1µg/ml) added. Images were taken using automatic autofocus with the green fluorescence channel of the JuLI-stage imaging device, without threshold modifications or any other manipulations. Bright field images correspond to the dark field pictures depicted. B. Example of the images (CTR or Cet-ZA) turned into black and white pictures (upper images) and analyzed with the Cell count plugin tool of the ImageJ2 computer program to measure fluorescence intensity evaluating the degree of grey nuances (middle images), after defining random 200 ROI throughout the well (lower images). C. Graphical representation of the analysis in B. Results are expressed as mean fluorescence intensity (MFI) as grey color intensity (arbitrary units, a.u.) and are the mean±SD from 3 experiments with different organoids and autologous Vδ2 T cells. *$p<0.001$.

Suppl. Figure 6. BTN3A1 or BTN2A1 expression and quantification in different CRC tumor areas. A. Histological definition of the adenomatous area at the luminal margin (LM-AD), luminal margin (LM), central tumor (CT), invasive margin (IM). B. Quantification (H-score) of BTN3A1 (left) or BTN2A1 (right) expression in the different areas of all CRC specimens. The mean±SD from 66 CRC patients is indicated. ***$p<0.0001$. C. IHC showing BTN3A1 expression in the different areas (LM-AD, LM, CT and IM) of one representative CRC. Enlargements of the squares in each image are also shown (arrows).