# DATA APPENDIX

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Nucleus CellBody		1. De	etection of c	listinct nuc	:lei.			
Ch.1								
1 MeanImage		,						
Mask Size [µm]		(	1) Image smo	othening				
2 Threshold								
Threshold [gray level]		(	2) Image bina	rization by ap	oplying a user	-defined thre	shold*	
3 OpeningCircle								
Diameter [µm]		(	3) Edges of de	etected objec	ts are smooth	nened		
4 FindMaximumDistance								
Minimum Point Distance [µm]		(	<ol> <li>Only object</li> </ol>					
Minimum Point Distance [µm] 4.0 +			adjacent objects are considered for further analysis to avoid					
Remove Size [µm] 0.1			double cou	nting. The ce	enters of these	e objects are	labeled.	
5 DilationCircle								
		(	5) The label o	f detected ob	jects is enlard	ged for better		
Diameter [µm] 5.0			(5) The label of detected objects is enlarged for better visualization					
6 DivideEachRegion		3						
			(6-7) All filtered objects are included					
7 DivideEachRegion < [3] OpeningCircle	]	(	,	,				
8 ExpandRegion3D < [6] DivideEachRegion , < [7] Divide	eEachReg	(	8) Labeled nu	clei are 3 dim	nensionally ex	panded to th	eir edges	
Nucleus CellBody		2. Se	gmentation	of adjacer	nt cells.			
Ch. 1			-	-				
1 MeanImage								
Mask Size [µm] 3.0 ф		(	1) Image smo	othening				
▼								
2 AdaptiveThreshold								
Object Size [µm] 20.0 🗘		(	2) Image bina	rization and a	application of	a size thresh	old for objects	
Object Detectivity		(	_,					
Object Detectivity 0.980								
3 DivideEachRegion			(3) Designation of distinct objects					
$\bigtriangledown$								
4 ExpandRegion3D < Nucleus Result ,			(4) Objects detected above (analysis 1 and 2) are expanded in 3D					
μm mode			to segment adjacent cells					
5 SizeFilter								
500.0								
Range [µm <sup>2</sup> or µm <sup>3</sup> ] { 100000.0			(5) Only objects greater than 500 μm <sup>3</sup> are considered					
( 1000000.0 -								
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10 Think is								
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### Appendix Figure S1. Image-based analysis to determine absolute organoid cell number

A) Analysis algorithm using the CQ1 software. The application 'Spheroid Structure' was first used to determine the organoid cell number based on DAPI signals (top) followed by segmentation of individual nuclei (bottom). \*: for optimal recognition the threshold was adjusted for each well separately.

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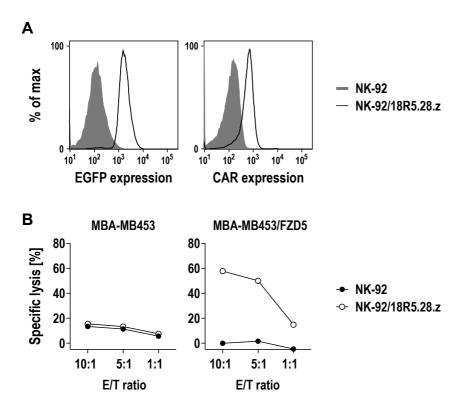
°S

B) Sample data for the analysis of one representative organoid. Maximum image projection (top left) and individual z stacks are shown. Automatically detected nuclei in each image are outlined in color. For cells spanning various z-stacks the color remains constant. Scale bars:  $50 \ \mu m$ .

Organoid Area Organoids+DilationCircle NK am Organoid	1. Detection of organoids.
Ch. 1 488nm BP525/50	
1 Meanimage	
Mask Size [µm]	(1) Image smoothening
2 Threshold	
Threshold [gray level] 160.0	(2) Image binarization by applying a user-defined threshold*
3 OpeningCircle	د ا
Diameter [µm] 25.0 🖕	
	(3-4) Noise reduction at the edge of detected objects*
Diameter [µm] 15.0	
Dianece (pari)	
5 DivideEachRegion	(5) All detected objects are designated as distinct objects
6 SizeFilter	
500.0	(6) Only objects greater than 500 $\mu$ m <sup>2</sup> are considered for further
Range [µm <sup>2</sup> or µm <sup>3</sup> ]	analysis
Organoid Area Organoids+DilationCircle NK am Organoid	2. Addition of a dilation sizely to detected expansion
Ch.1 488nm BP525/50	2. Addition of a dilation circle to detected organoids.
1 MeanImage	a
Mask Size [µm]	
$\overline{\nabla}$	
2 Threshold	
Threshold [gray level] 160.0 🗘	
→ → → → → → → → → → → → → → → → → → →	(1-4) As described above
Diameter [µm] 25.0	
4 ClosingCircle	
Diameter [µm] 15.0 🗘	
5 DilationCircle	
Diameter [µm] 50.0 _	(5) Detected objects are expanded by addition of a dilation circle.
	(6) All expanded objects are designated as distinct objects and
6 DivideEachRegion	(6) All expanded objects are designated as distinct objects and assigned an individual object ID
Organoid Area Organoids+DilationCircle NK am Organoid	3. Counting of NK-92 cells within the dilated organoid area.
Ch. 3 640nm BP685/40	
1 MeanImage	(1) Image smoothening
Mask Size [µm]	(T) mage on outloning
2 Threshold	
Threshold [gray level] 250.0	(2) Image binarization by applying a user-defined threshold*
3 ErosionCircle	د ا
Diameter [µm] 8.0 🌲	
4 OpeningCircle	(3-4) The area of detected objects is reduced to separate individual objects
	Objecta
Diameter [µm]	
5 FindMaximumDistance	(E) Objects were reduced to points with measimum distance from their
Minimum Point Distance [µm]	(5) Objects were reduced to points with maximum distance from their edges. Only objects with a user defined minimum distance to adjacent
Remove Size [µm]	objects are considered for further analysis to avoid double counting
6 DilationCircle	(6) Objects detected by then are assigned a user-definded artifical
Diameter [µm] 0.1 🗘	diameter. Objects of this size are further recognized and counted
7 DivideEachRegion	(7) All detected objects are designated as distinct objects
8 SizeFilter	(8) Size filtering is adjusted to detect objects of the size set in (6)
Range [µm² or µm²]	
100000000.0 C	(9) Limiting the detection and counting of objects to the expanded
9 AndRegion , < Organoids+DilationCircle Result	organoid area (Only objects detected in (8) which are additionally
, see organoluse Dilationarcie Result	located in a dilated organoid area (Analysis level 2) are counted)

# Appendix Figure S2. Image-based analysis of organoid area and NK-cell recruitment

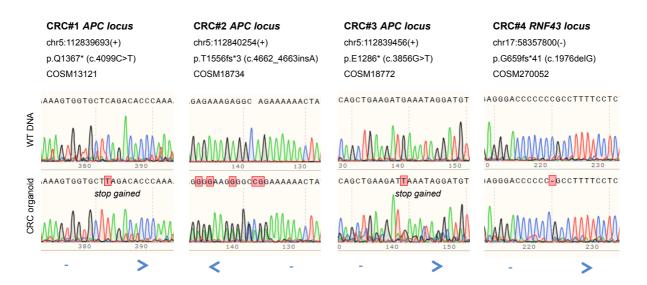
Analysis algorithm using the CQ1 software. First the organoid area was determined from GFP signals (top panel). Then a recruitment area was defined by expanding the organoid areas with a constant diameter of 50  $\mu$ m (middle panel). In this expanded area all CD45-APC positive NK cells were counted using a third level of analysis (bottom panel). \*: for these steps adjustments were set for each well separately.



Appendix Figure S3. Generation and characterization of pan FZD-CAR NK-92 cells

A) Expression of the FZD-CAR and the co-expressed GFP in transduced NK-92 cells was studied by flow cytometry. CAR expression was detected with Myc-tag-specific antibody. Parental NK-92 cells were included as control.

B) Cytotoxic activity of FZD-CAR NK-92 cells against MDA-MB453 cells stably expressing FZD5. Parental MDA-MB453 and NK-92 cells were used as controls.



# Appendix Figure S4. RNF43 and APC genotyping of human CRC organoids

Sanger sequencing spectrograms using genomic DNA from WT (top) and CRC organoids (bottom) identifies damaging coding mutations in the *APC* locus (CRC#1-3) or in *RNF43* (CRC#4). For all lines the *APC* mutation cluster region (1132 bp) and for *RNF43* a recurrently mutated region (1244bp; Giannakis et al., 2014) was analyzed and no additional mutations were identified. Genomic coordinates (hg38) and protein consequences and COSMIC mutation IDs are indicated. The synthesis direction of sequencing is indicated by the blue arrow.