

Supplementary information

Radiobiological and immunomodulatory mechanisms of CAIX-targeted α therapy combined with immune checkpoint inhibition in hypoxic tumors

Sylvia T.M. Wenker^{1,2}, Rowan Wuestenenk^{1,2}, Janneke D.M. Molkenboer-Kuenen¹, Vera Mekers², Kris Raaijmakers², Marleen Ansems², Paul Span², Johan Bussink², Sandra Heskamp¹, Sanne A.M. van Lith¹

¹Department of Medical Imaging: Nuclear Medicine, Radboudumc, Nijmegen, The Netherlands

²Department of Radiation Oncology: Radiotherapy & Oncoimmunology laboratory, Radboudumc, Nijmegen, The Netherlands

Corresponding author: Sanne van Lith, Geert Grooteplein Zuid 10, +31 24 36 14511, sanne.vanlith@radboudumc.nl

Supplemental Methods

Conjugation, radiolabeling and quality control

DOTA-conjugated anti-CAIX monoclonal antibody (clone: MSC3, TAB-1458CL, Creative Biolabs, New York, USA), i.e. DOTA-MSC3, was prepared as described previously [1]. DOTA-MSC3 was labelled with ²²⁵Ac at a molar activity of 100 kBq/nmol. Labelling efficiency was determined using instant thin-layer chromatography as described previously [1-4]. Radiochemical purity exceeded 95%. *In vitro* binding characteristics of [²²⁵Ac]Ac-DOTA-MSC3 have been described previously [1].

Cell culture

B16F10-OVA murine melanoma cells were kindly provided by Dr. K.L. Rock, Dana-Farber Cancer Institute, Boston, USA [5], and cells were cultured in MEM (cat# 21575-022 Gibco, Thermo Fisher Scientific, Waltham, USA) supplemented with 5% fetal calf serum, 1 mg/mL geneticin (G418), 1.5 mg/mL sodium bicarbonate, 1.5% MEM vitamins, 1% antibiotic/antimycotic, 110 µg/mL sodium pyruvate, 1% nonessential amino acids, 0.05 mM β-mercaptoethanol and 60 µg/mL hygromycin (all Gibco) at 37°C in an incubator containing 5% CO₂. For tumour cell inoculation, cells were passaged <3 times post thawing. B16F10-OVA cells express CAIX when cultured under hypoxic conditions [6].

Animal experiment

Mice were housed in individually ventilated cages with *ad libitum* access to animal chow and water. Before starting the experiment, animals were allowed to acclimatize for at least one week. For each animal experiment, the number of animals inoculated with B16F10-OVA tumour cells was determined based on an expected tumour take of 75%. Tumours were measured 3x/week with a calliper and tumour volumes (mm³) were calculated using the following formula: $4/3 * \pi * \text{length}/2 * \text{height}/2 * \text{width}/2$. Mice were sacrificed by cervical dislocation at the end of the experiment or when humane endpoints (HEPs) were reached. Due to practical and radiation safety reasons, mice were housed according to the treatment groups. Tumour measurements and examining the mice were assessed by biotechnicians blinded for group allocation. The investigator was not blinded during treatment injections, dissection and analysis. An *a priori* sample size calculation was based on observed treatment effects and its variation (as found in a pilot experiment), a significance level of 5%, power of 80% and a two-way ANOVA. Allocation to the treatment groups was block-randomized by tumour size, using a random number generator.

IF staining of γH2AX, caspase-3, PD-L1, CD45, CD8, F4/80, FOXP3 and Ly6G

FFPE tumour sections (4 µm) were mounted on FLEX IHC Microscope Slides (#K802021-2, Agilent Technologies, Santa Clara, USA). Cross-sections from the center of the tumours (one section per mouse) were stained for the following markers: phosphorylated histone 2A family member X (γH2AX), apoptosis (caspase-3), intrinsic inhibition of T cells (programmed death ligand-1 (PD-L1)), immune cells (cluster of differentiation 45 (CD45)), cytotoxic T-cells (cluster of differentiation 8 (CD8)), macrophages (F4/80), regulatory T-cells (Treg) (Forkhead box P3 (FOXP3)) and neutrophils (lymphocyte antigen 6 family member G (Ly6G)). Sections were deparaffinated and dehydrated, followed by antigen retrieval in 10 mM sodium citrate pH 6.0 (for γH2AX, caspase-3, PD-L1, CD45, F4/80 and Ly6G) or Tris-borate-EDTA buffer solution (TBE) (for CD8 and FOXP3). Between consecutive steps of the staining process, sections were rinsed 3 times in Tris-buffered saline (25 mM TRIS-HCl + 150 mM NaCl, pH 7.5) with 0.05% Tween-20 (Merck, Rahway, USA) (TBST). Endogenous peroxidase activity was blocked at room temperature (RT) for 10 min incubation with 3% H₂O₂ (Merck) in PBS and non-specific binding was blocked for 10 min incubation with TBST + 1% bovine serum albumin (BSA) (Sigma-Aldrich). Additionally, for F4/80 and FOXP3, slides were blocked for endogen biotin using a Biotin/avidin blocking kit (SP2001, Vector Laboratories, Newark, USA), before blocking with TBST + 1% BSA. After the blocking steps, sections were incubated at RT for 1 h with primary anti-γH2AX antibody (1:500 dilution, RRID:AB_2118009, #9718, Cell Signaling Technology, Beverly, USA), anti-cleaved-caspase-3 antibody (1:1000 dilution, RRID:AB_2341188, #9661, Cell Signaling Technology), anti-PD-L1 antibody (1:500 dilution, RRID:AB_354540, #AF1019, R&D systems, Minneapolis, USA), anti-CD45 antibody

(1:150 dilution, RRID:AB_2799780, #70257, Cell Signaling Technology), anti-CD8 antibody (1:1000 dilution, RRID:AB_2890649, #ab217344, Abcam, Cambridge, UK), anti-F4/80 antibody (1:1000 dilution, RRID:AB_467558, #14-4801-82, Thermo Fisher Scientific), anti-FOXP3 antibody (1:800 dilution, RRID:AB_467576, #14-5773-82, Thermo Fisher Scientific), or anti-Ly6G antibody (1:16000 dilution, RRID:AB_1107721, #BP0075-1, BioXCell), all in TBST + 1% BSA. For biotinylated secondary antibodies, slides were incubated with rabbit anti-rat Biotin (RRID:AB_10015300, #BA-4001, Vector Laboratories) (1:200 dilution in TBST + 1% BSA for F4/80 and 1:400 dilution in PBS + 1% BSA for FOXP3) or rabbit anti-goat Biotin (RRID:AB_228393, #31732, Thermo Fisher Scientific) (1:500 dilution in TBST + 1% BSA for PD-L1) followed by Vectastain ABC-HRP kit according to manufacturer's instructions (PK-6100, Vector Laboratories). For non-biotinylated slides, slides were incubated with secondary antibody at RT for 30 min incubation with Opal polymer anti-mouse with anti-rabbit HRP (RRID:AB_2890927, #ARH1001EA, Akoya Biosciences, Marlborough, USA) in TBST + 1% BSA or for Ly6G with goat anti-rat (RRID:AB_258429, #A9037, Sigma-Aldrich, 1:200 dilution in TBST + 1% BSA). Then, for all markers, followed by Opal-620 fluorophore working solution (1:200, #FP1495001KT, Akoya Biosciences) according to manufacturer's instructions. Again, antigen retrieval in 10 mM sodium citrate pH 6.0 (for all) was performed as described above. Slides were blocked again at RT for 10 min incubation with TBST + 1% BSA. Immediately after, sections were incubated with primary anti-CAIX antibody (RRID:AB_10003398, #NB100-417, Novus Biologicals, Centennial, USA) (1:2000-1:8000 dilution in 1% BSA). Slides were incubated with secondary antibody at RT for 30 min with Opal polymer anti-mouse, anti-rabbit HRP (RRID:AB_2890927, #ARH1001EA, Akoya Biosciences), followed by incubation with Opal-520 fluorophore working solution (1:200-1:250 dilution, #FP1487001KT, Akoya Biosciences). Another antigen retrieval step was performed with 10 mM sodium citrate (for all) and slides were incubated at RT for 2 min with DAPI (1:5000 solution in PBS, #D1306, Thermo Fisher Scientific) and mounted a cover slip (1.5 mm, #631-0147, VWR International, Radnor, USA) using Fluoromount (#F2680, Sigma-Aldrich).

IF staining of cGAS expression

Snap-frozen tumour sections (4 µm) were mounted on FLEX IHC Microscope Slides. One tumour cross-section from the center of the tumours per mouse was used for IF for cyclic GMP-AMP synthase (cGAS): a DNA sensing immune activation marker. Sections were fixated with 4% paraformaldehyde at RT for 10 min. Slides were rehydrated and incubated with 5% normal donkey serum in primary antibody diluent (PAD, Biolegend, San Diego, USA) at RT for 30 min and immediately incubated with primary anti-cGAS antibody (1:200 dilution, RRID:AB_2799008, #31659, Cell Signaling Technology) at 4°C overnight. Between consecutive steps of the staining process, sections were rinsed 3 times in PBS. Then, slides were incubated with goat anti-rabbit FabCy3 (1:400 dilution, RRID:AB_2313593, Jackson ImmunoResearch, West Grove, USA) in PAD at 37°C for 45 min, followed by donkey anti-goatCy3 (1:400 dilution, RRID:AB_2340413, Jackson ImmunoResearch) in PAD at 37°C for 45 min. Sections were incubated with primary anti-CAIX antibody (1:500 dilution) in PAD at 4°C overnight. Next, donkey anti-rabbit Alexa488 (1:100 dilution, RRID:AB_2535792, Thermo Fisher Scientific) was incubated 37°C for 45 min, followed by Hoechst (1:2000 dilution of 1 mg/ml) at RT for 5 min and mounted a cover slip using Fluoromount.

Supplemental Tables

Suppl. Table 1: Overview of mice excluded because mice reached humane endpoint (HEP) before endpoint. Table adapted from [1]. HEPs include tumor ulceration and severe clinical deterioration.

Group	# Mice reached HEP
PBS 5 days p.i.	1
PBS 8 days p.i.	2
ICI 8 days p.i.	2
CAIX-TAT + ICI 8 days p.i.	2
Total	7

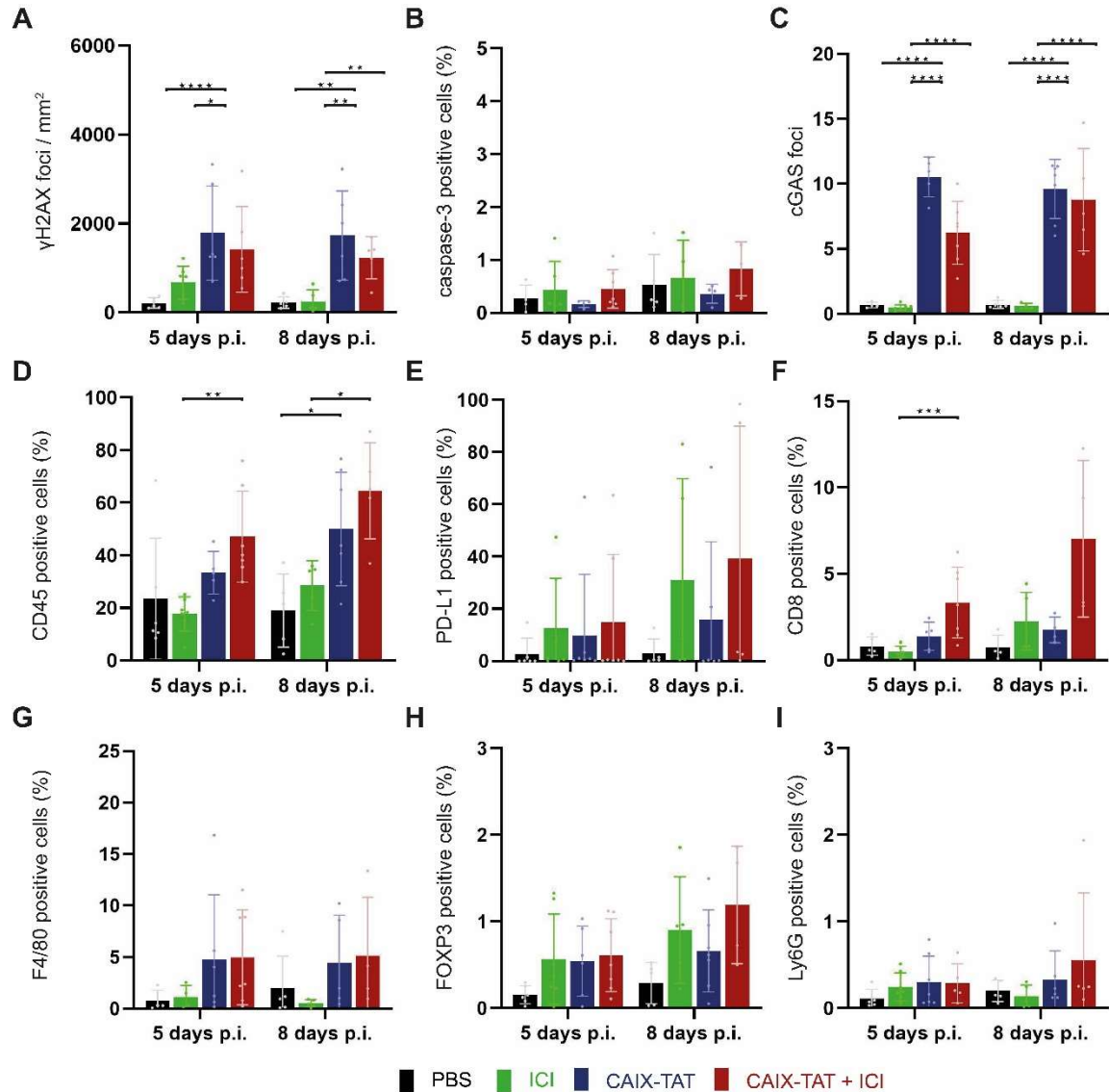
Suppl. Table 2: Overview of total number of mice included per marker in IF quantification. Exclusion was because tumors were too small, tumor sections were damaged, or there was limited CAIX expression. For these reasons, baseline group was excluded in all analysis.

		Markers								
	Group	γ H2AX	caspase-3	PD-L1	cGAS	CD45	CD8	FOXP3	F4/80	Ly6G
5 days p.i.	PBS	6	4	6	4	6	5	6	4	5
	ICI	7	6	7	7	7	6	7	4	7
	CAIX-TAT	6	5	7	5	5	5	6	6	7
	CAIX-TAT + ICI	6	7	7	7	7	7	7	7	5
8 days p.i.	PBS	5	5	5	5	5	4	5	5	5
	ICI	5	4	5	5	5	5	5	5	5
	CAIX-TAT	6	4	6	7	7	4	7	5	6
	CAIX-TAT + ICI	5	3	5	5	5	4	4	4	5

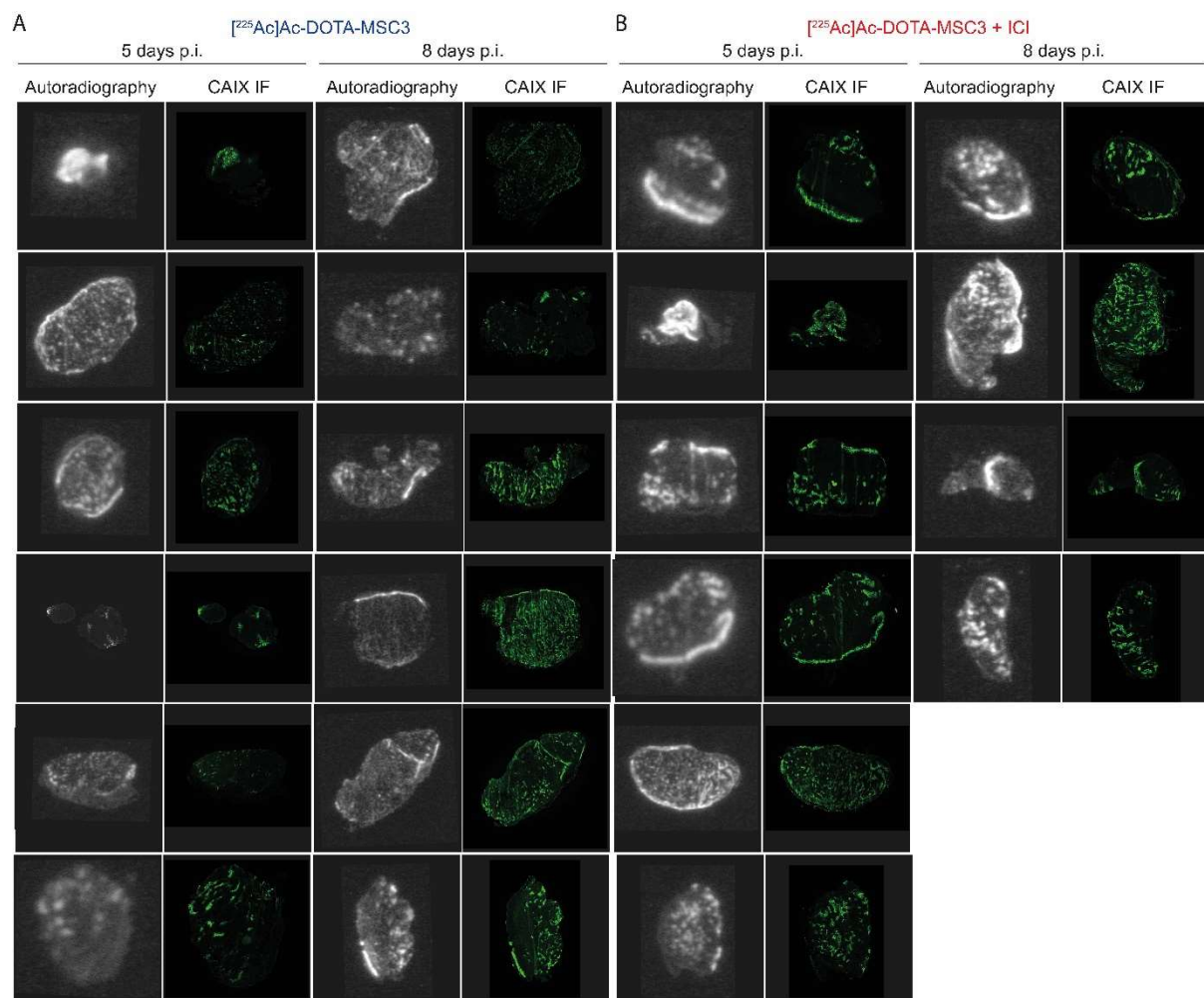
Suppl. Table 3: Ex vivo biodistribution analysis of [^{225}Ac]Ac-DOTA-MS3 in B16F10-OVA tumor-bearing C57BL/6J mice at 5 and 8 days p.i.. Data represent mean tissue uptake (in %IA/g $\pm$ SD), *Signal in gamma counter was below detection limit. At day 8, only tumor, liver, kidneys and lung were analyzed due to practical reasons.

	5 days p.i.		8 days p.i.	
	[^{225}Ac]Ac-DOTA-MS3 (n=7)	[^{225}Ac]Ac-DOTA-MS3 + ICI (n=7)	[^{225}Ac]Ac-DOTA-MS3 (n=7)	[^{225}Ac]Ac-DOTA-MS3 + ICI (n=5)
Tumor	22.0 $\pm$ 12.5	17.5 $\pm$ 4.4	8.0 $\pm$ 4.4	15.5 $\pm$ 9.2
Blood	2.7 $\pm$ 1.4	2.3 $\pm$ 0.3		
Liver	25.6 $\pm$ 3.8	26.1 $\pm$ 4.3	26.7 $\pm$ 2.9	25.8 $\pm$ 3.6
Kidneys	3.6 $\pm$ 0.4	3.5 $\pm$ 0.4	2.2 $\pm$ 0.2	2.2 $\pm$ 0.4
Heart	1.8 $\pm$ 0.5	1.5 $\pm$ 0.1		
Lung	10.4 $\pm$ 3.4	10.0 $\pm$ 1.7	3.2 $\pm$ 0.8	4.1 $\pm$ 0.8
Thymus	*	*		
Brown fat	*	*		
Salivary gland	2.0 $\pm$ 0.3	1.6 $\pm$ 0.3		
Stomach	6.5 $\pm$ 1.2	6.3 $\pm$ 1.5		
Pancreas	2.8 $\pm$ 0.5	2.7 $\pm$ 0.3		
Spleen	10.6 $\pm$ 2.3	9.6 $\pm$ 0.5		
Duodenum	14.2 $\pm$ 4.6	12.8 $\pm$ 3.3		
Jejunum	7.2 $\pm$ 1.5	7.2 $\pm$ 1.1		
Ileum	4.1 $\pm$ 0.7	3.7 $\pm$ 0.8		
Colon	2.7 $\pm$ 0.6	2.8 $\pm$ 0.5		
Lymph nodes	*	*		
Bone marrow	*	*		
Bone	*	*		
Muscle	*	*		
Tail	1.2 $\pm$ 0.1	1.3 $\pm$ 0.2		

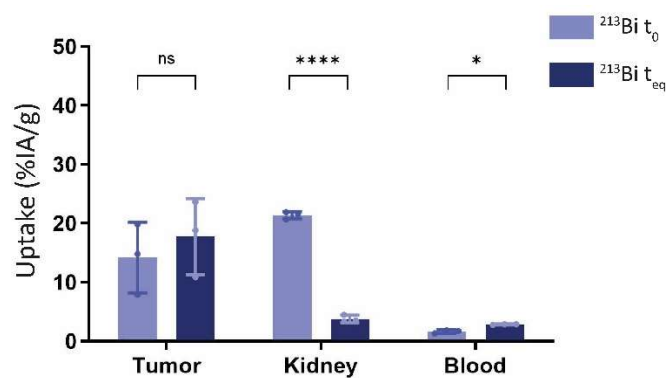
Supplemental Figures



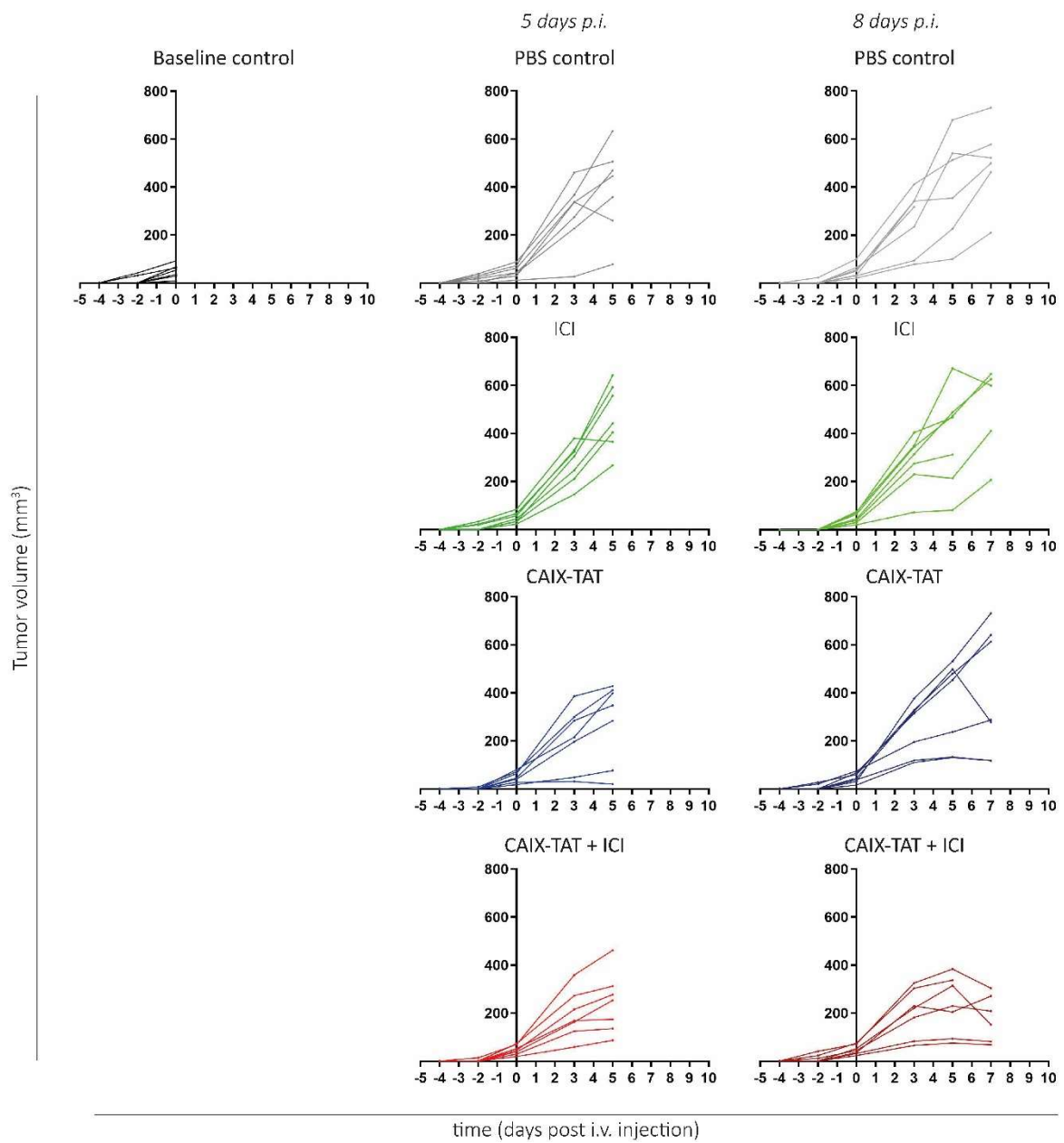
Suppl. Figure 1: IF analysis in B16F10-OVA tumors (CAIX⁺ and CAIX⁻ averaged) from mice treated with PBS, ICI, CAIX-TAT and CAIX-TAT+ ICI at 5 and 8 days p.i.. **A** γ H2AX foci / mm², **B** caspase-3 positive cells (%), **C** cGAS foci, **D** CD45 positive cells (%), **E** PD-L1 positive cells (%), **F** CD8 positive cells (%), **G** F4/80 positive cells (%), **H** FOXP3 positive cells (%) and **I** Ly6G positive cells (%). Differences between CAIX-TAT and PBS and ICI control and CAIX-TAT+ ICI and ICI alone are tested on each time point by one-way ANOVA Šidák multiple comparisons test, using a log or logits transformation. * = adjusted $p < 0.05$, ** = adjusted $p < 0.01$, *** = adjusted $p < 0.001$, **** = adjusted $p < 0.0001$. For all comparisons, treatment-tumor area interaction was not significant.



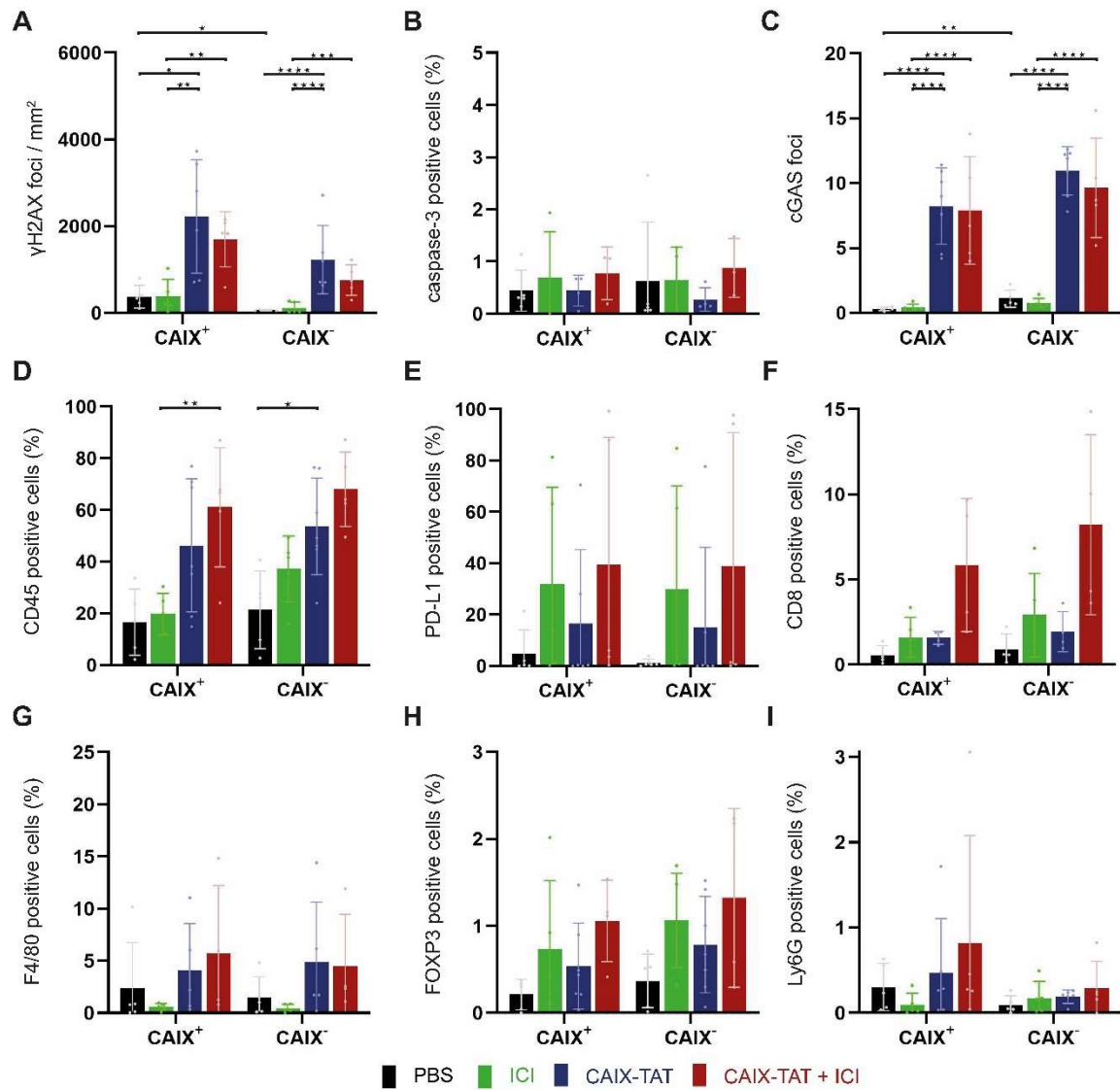
Suppl. Figure 2: Ex vivo autoradiography and CAIX IF images of B16F10-OVA tumor sections at 5 and 8 days **A** after $[^{225}\text{Ac}]\text{Ac-DOTA-MSD3}$ and **B** after $[^{225}\text{Ac}]\text{Ac-DOTA-MSD3} + \text{ICI}$ therapy. These images are in addition to Figure 2C.



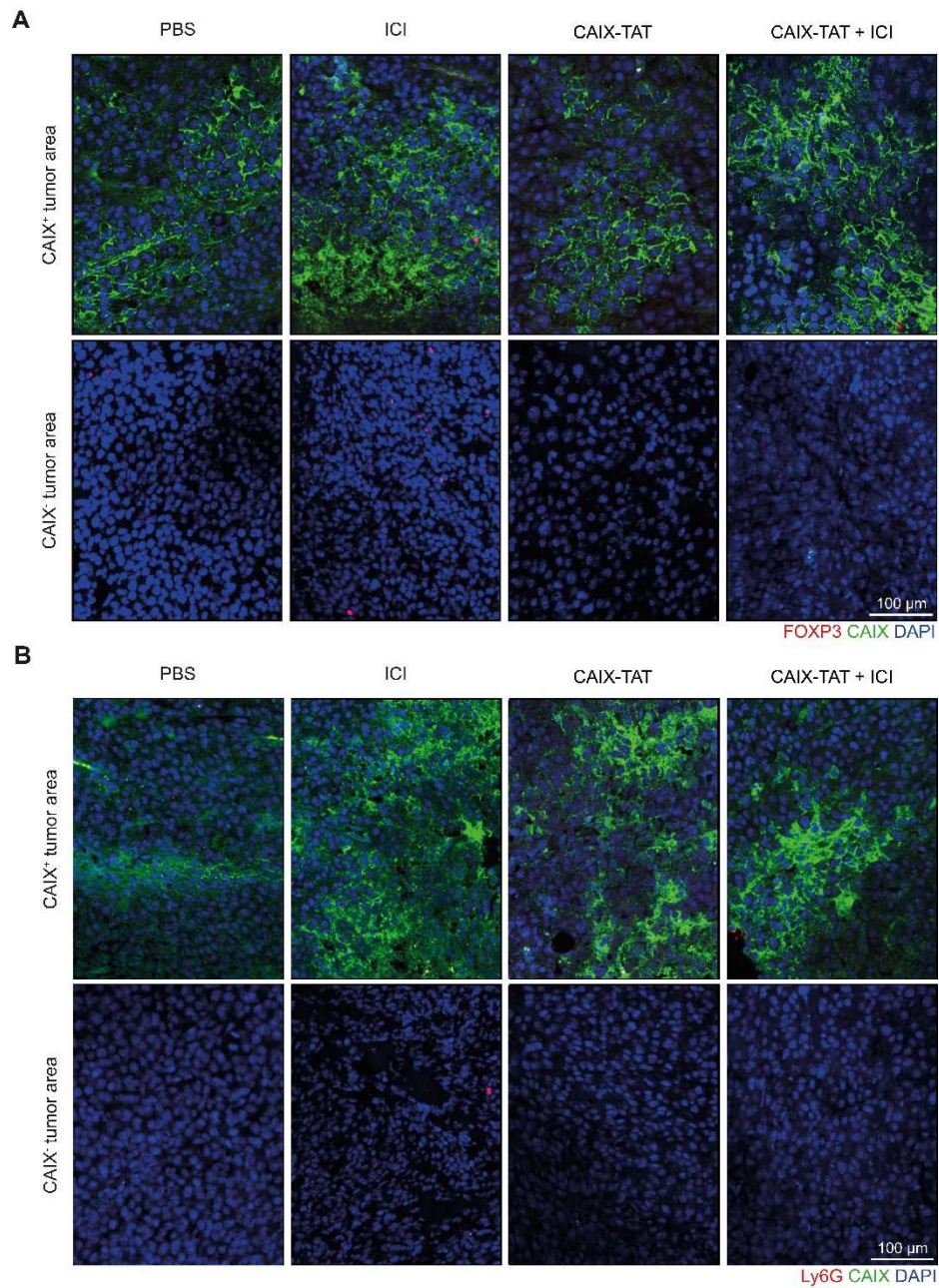
Suppl. Figure 3: The calculated uptake (%IA/g) of ^{213}Bi at time of dissection (t_0) and the uptake at equilibrium state (t_{eq}) in tumor, kidney and blood in B16F10-OVA tumor-bearing mice at 5 days p.i.. Data represent mean tissue uptake (in %IA/g) with SD. Differences between t_0 and t_{eq} was tested using a Welch's t-test with *: $p < 0.05$, ****: $p < 0.0001$ and n.s.=not significant.



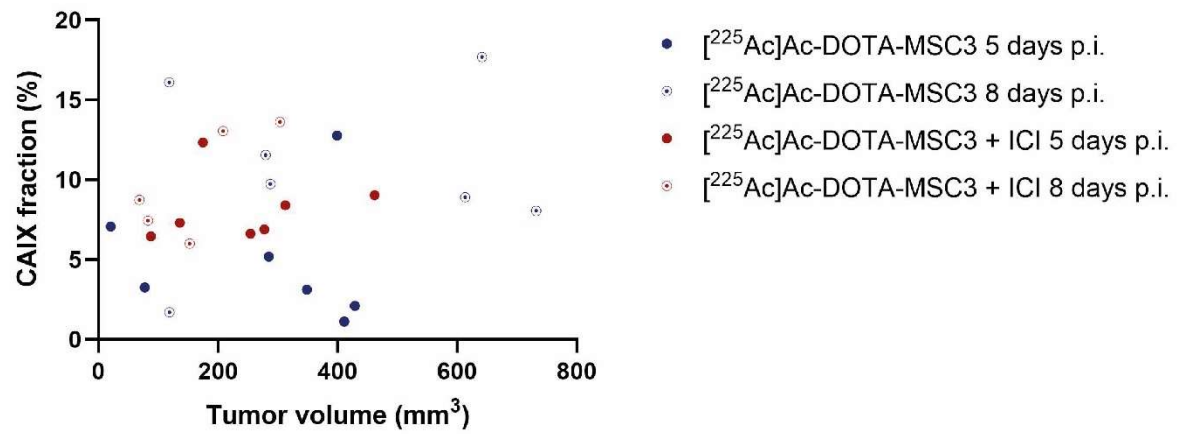
Suppl. Figure 4: Tumor growth curves of all individual mice in control groups (Baseline, PBS or ICI alone) and mice treated with CAIX-TAT or CAIX-TAT + ICI. Body weight curves are shown in Suppl. Figure 8.



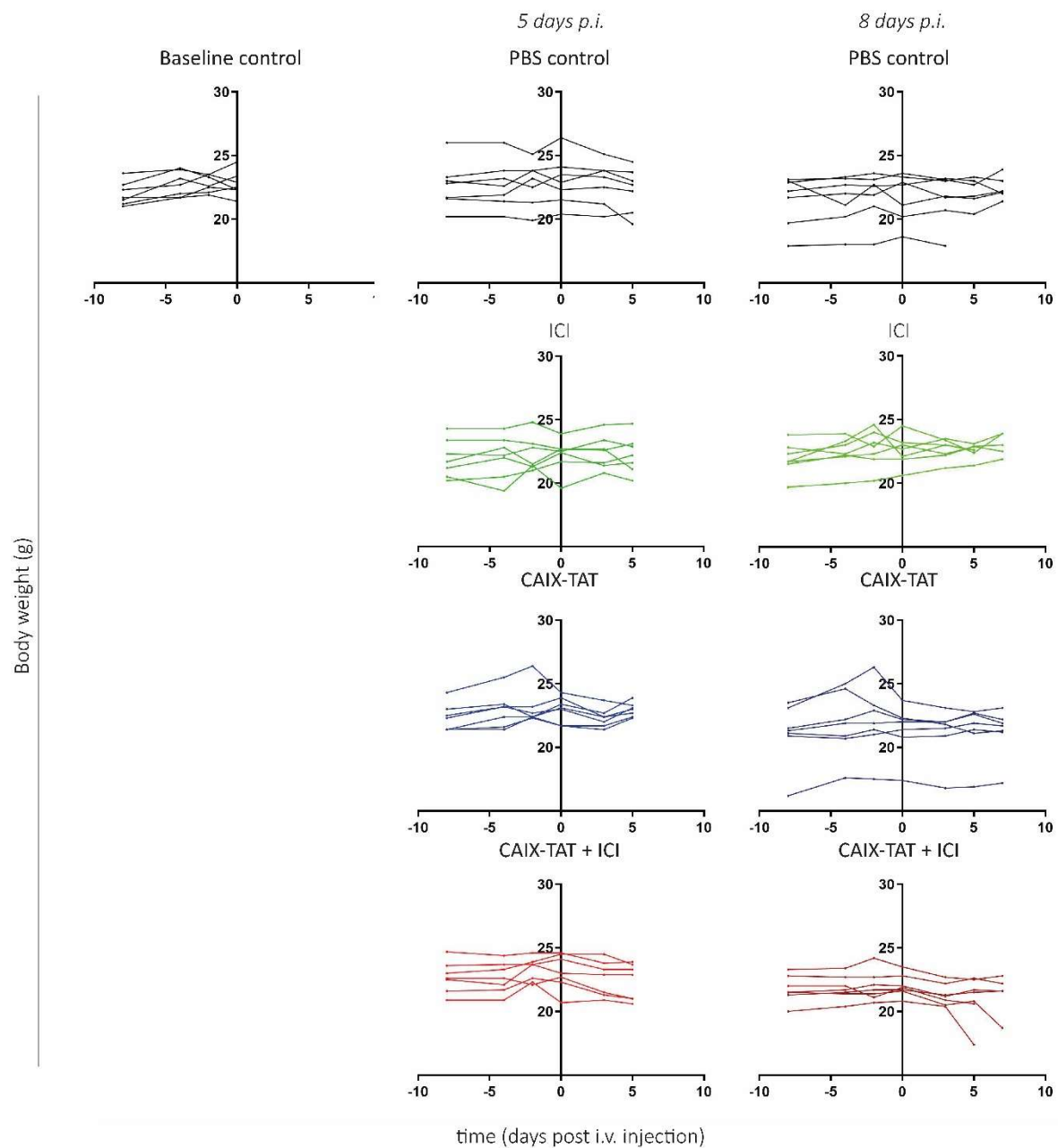
Suppl. Figure 5: IF analysis of B16F10-OVA tumors from mice treated with PBS, ICI, CAIX-TAT and CAIX-TAT + ICI at 8 days p.i. in CAIX⁺ and CAIX⁻ tumor areas. **A** γ H2AX foci / mm², **B** caspase-3 positive cells (%), **C** cGAS foci, **D** CD45 positive cells (%), **E** PD-L1 positive cells (%), **F** CD8 positive cells (%), **G** F4/80 positive cells (%), **H** FOXP3 positive cells (%) and **I** Ly6G positive cells (%). Differences between CAIX-TAT and PBS and ICI control and CAIX-TAT + ICI and ICI alone are tested on each time point by one-way ANOVA Šídák multiple comparisons test, using a log or logits transformation. * = adjusted $p < 0.05$, ** = adjusted $p < 0.01$, *** = adjusted $p < 0.001$, **** = adjusted $p < 0.0001$. For all comparisons, treatment-tumor area interaction was not significant.



Suppl. Figure 6: FOXP3 and Ly6G IF analysis of B16F10-OVA tumors from mice treated with PBS, ICI, CAIX-TAT and CAIX-TAT + ICI at 5 days p.i. in CAIX⁺ and CAIX⁻ tumor areas. **A** Representative IF images of FOXP3 (red), CAIX (green) and DAPI (blue). **B** Representative IF images of Ly6G (red), CAIX (green) and DAPI (blue).



Suppl. Figure 7: Correlation of CAIX fraction and tumor volume (in mm³) for the different groups. CAIX fraction was based on the CAIX-expression in % of the tumor section and tumor volume was measured with a caliper and tumor volumes (mm³) were calculated using the following formula: $\frac{4}{3} \cdot \pi \cdot \text{length}/2 \cdot \text{height}/2 \cdot \text{width}/2$.



Suppl. Figure 8: Body weight curves of all individual B16F10-OVA tumor-bearing mice of baseline group or after treatment with PBS, ICI, CAIX-TAT or CAIX-TAT + ICI at 5 and 8 days p.i..

References

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