Supplemental Material

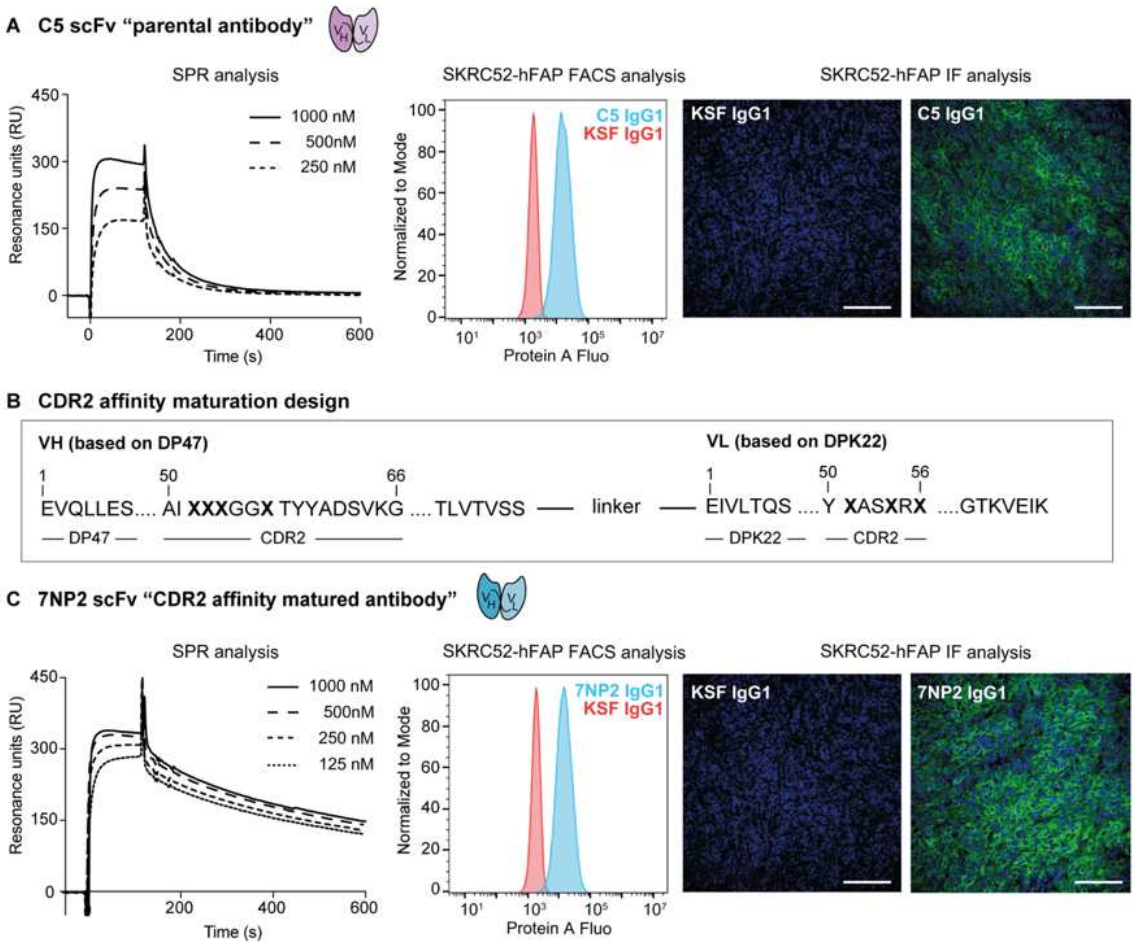

Figure S1. Characterization of antibodies targeting human FAP

A. Characterization of scFv(C5) selected from synthetic phage display library: BIAcore sensorgrams of monomeric scFvs on hFAP coated SA chip; Flow cytometry analysis on SKRC52-hFAP cells with C5 in IgG1 format (Protein A-AlexaFluor 488); Microscopic fluorescence analysis on SKRC52-hFAP tumor section detected with IgG1(C5)-FITC (green, AlexaFluor 488), cell nuclei were counterstained with DAPI (blue). Representative pictures of the samples were taken 20x magnification, scale bars = 100 µm.

B. Schematic representation of scFv(C5) CDR2 affinity maturation design of the phage library.

C. Characterization of scFv(7NP2) from CDR2 affinity maturation library of C5: BIAcore sensorgrams of monomeric scFvs on hFAP coated SA chip; Flow cytometry analysis on SKRC52-hFAP cells with 7NP2 in IgG1 format (Protein A-AlexaFluor 488); Microscopic fluorescence analysis on SKRC52-hFAP tumor section detected with IgG1(7NP2)-FITC (green, AlexaFluor 488), cell nuclei were counterstained with DAPI (blue). Representative pictures of the samples were taken 20x magnification, scale bars = 100 µm.

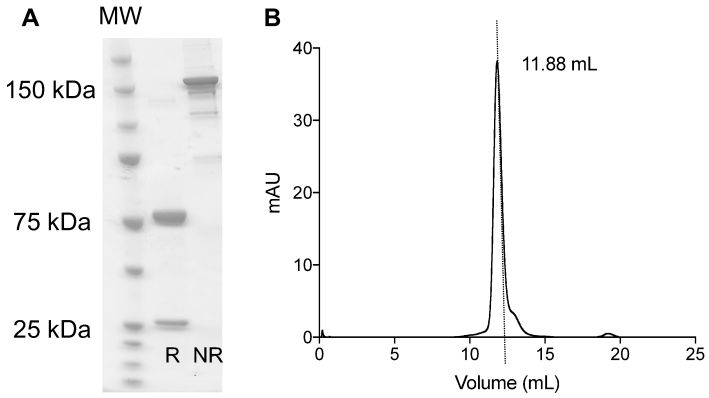

Figure S2. 7NP2 IgG1 biochemical characterization.

A. SDS-page, 10% gel in reducing (R) and non-reducing (NR) conditions of purified 7NP2 IgG1; B. Size exclusion chromatogram of 7NP2 IgG1.

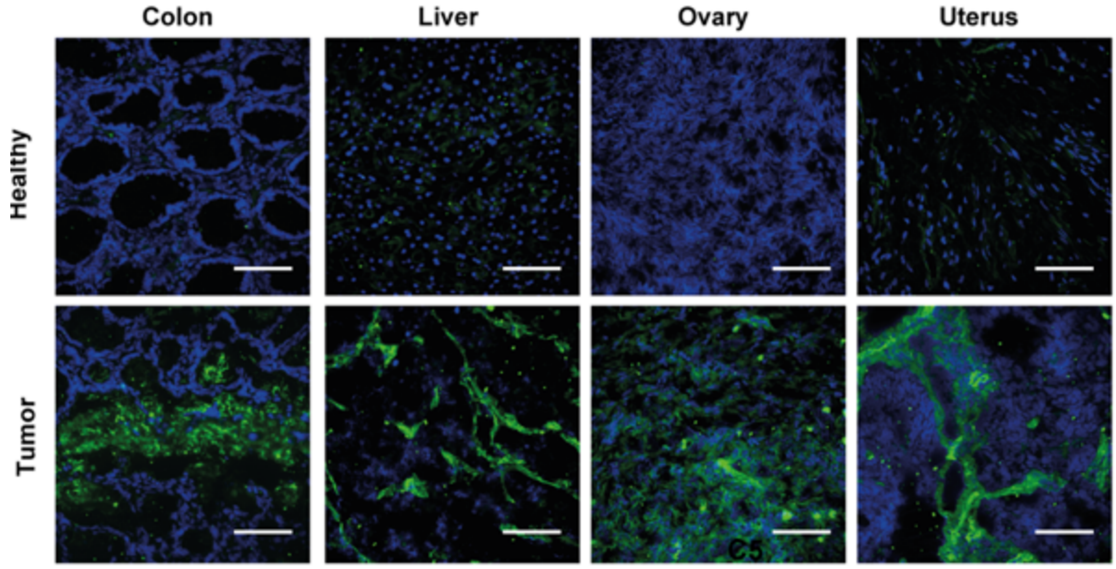

Figure S3. Immunofluorescence analysis of hFAP expression on tissue human microarray with IgG1(7NP2)-FITC.

Microscopic fluorescence analysis of human FAP expression on tissue microarray detected with IgG1(7NP2)-FITC. Cryosections were stained with anti-FITC (green, AlexaFluor 488); cell nuclei were stained with DAPI (blue). 20x magnification, scale bars = 100 μm.

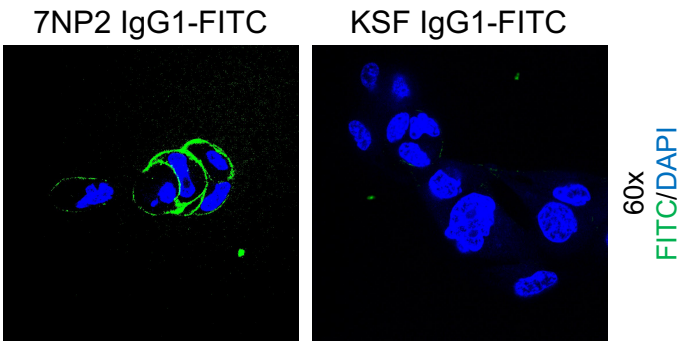

Figure S4. Confocal microscopy images after exposure to 7NP2 IgG1-FITC on SKRC52-hFAP cells.

SKRC52-hFAP were seeded into 4-well cover slip chamber plates (Sarstedt) at a density of 10^4 cells per well in RPMI medium (1 mL, Invitrogen) supplemented with 10% FCS, AA and HEPES (10 mM) and allowed to grow for 24 hours under standard culture conditions. Hoechst 33342 nuclear dye (Invitrogen) was used to stain nuclear structures. The culture medium was replaced with fresh medium containing 7NP2 IgG1-FITC and KSF IgG1-FITC at a concentration of 10 μg/mL. Randomly selected colonies imaged 1 hour after incubation on a SP8 confocal microscope equipped with an AOBS device (Leica Microsystems). 7NP2 was mainly bound to the SKRC52-hFAP cell surface showing lack of internalization (left panel). KSF IgG1-FITC (right panel) was used as negative control for the experiment, and it did not show any binding to the cell membrane.

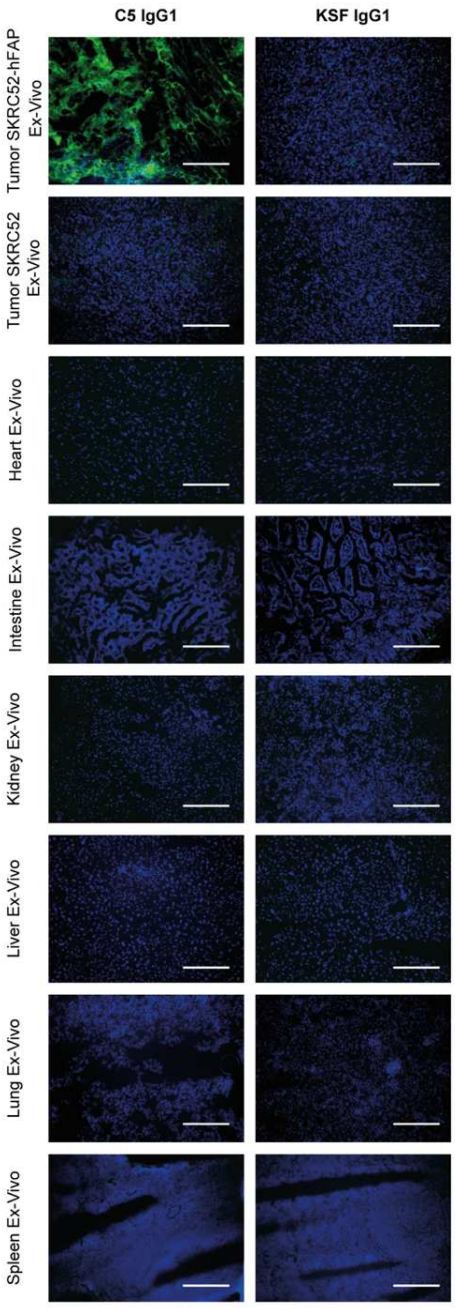

Figure S5. Immunofluorescence-based biodistribution analysis with IgG1 (C5).

Microscopic fluorescence analysis of tumor-targeting performance of IgG1(C5) on SKRC52-hFAP tumor and organs from BALB/c nude mice. One hundred micrograms of IgG1(C5)- FITC or IgG1(KSF)-FITC (negative control) were injected intravenously into the lateral tail vein and mice were sacrificed 24 hours after injection. Tumor and organs were excised and embedded in cryoembedding medium; cryostat sections were stained with ant-FITC (green, AlexaFluor 488) and DAPI (blue). Representative pictures of the samples were taken at 20x magnification, scale bars = 100 μm.

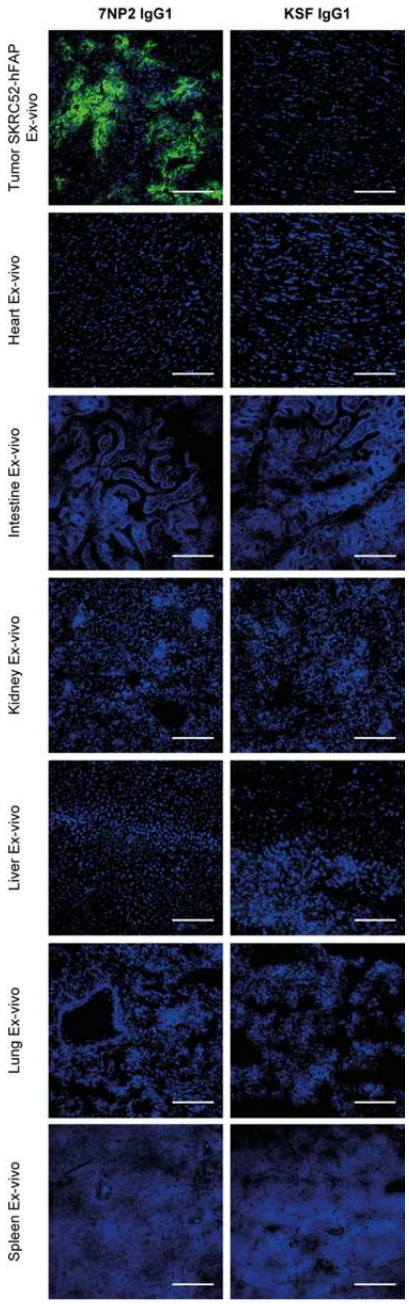

Figure S6. Immunofluorescence-based biodistribution analysis with 7NP2 IgG1.

Microscopic fluorescence analysis of tumor-targeting performance of 7NP2 IgG1 on SKRC52-hFAP tumor and organs from BALB/c nude tumor-bearing mice. One hundred micrograms of 7NP2 IgG1-FITC or KSF IgG1-FITC (negative control) were injected intravenously into the lateral tail vein and mice were sacrificed 24 hours after injection. Tumor and organs were excised and embedded in cryoembedding medium; cryostat sections were stained with ant-FITC (green, AlexaFluor 488) and DAPI (blue). Representative pictures of the samples were taken at 20x magnification, scale bars = 100 μm.

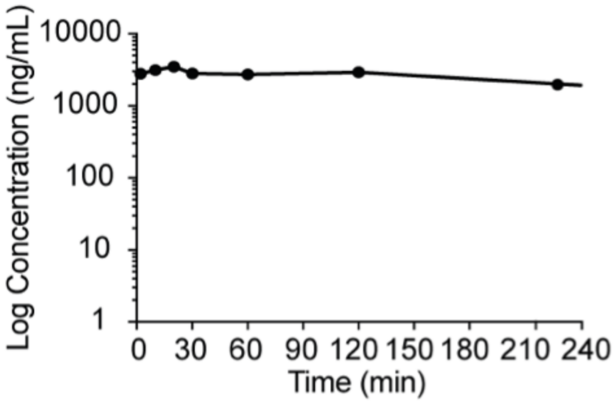

Figure S7. Pharmacokinetics in *Cynomolgus* monkeys.

Pharmacokinetics were evaluated in one *Cynomolgus* monkey injected once at the dose of 0.1 mg/kg of IgG1(7NP2). Blood samples were collected before dosing and at 2, 10, 20 and 30 min and 1, 2, and 4 h after injection. IgG1(7NP2) concentrations in monkey serum were assessed by ELISA. Briefly, 100 nM of recombinant hFAP were coated on 96 well plates overnight at 4°C. After a blocking step, serum samples were incubated for 1 h and detected with anti-human IgG (Fc specific) – Peroxidase antibody (Sigma A0170).

Figure S8. Amino acid sequence of mIL12-7NP2. Sequence from PCT/EP2022/060776

MWELEKDVYVVEVDWTPDAPGETVNLTCDTPEEDDITWTSDQRHGVIGSGKTLTITVKEFLDAGQYTCHKGGETL
SHSHLLLHKKENGIWSTEILKNFKNKTFLKCEAPNYSGRFTCSWLVQRNMDLKFNIKSSSSSPDSRAVTCGMASL
SAEKVTLDQRDYEKYSVSCQEDVTCPTAEETLPIELALEARQQNKYENYSTSFFIRDIIKPDPPKNLQMRPLKNS
QVEVSWEYPDSWSTPHSYFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVERTSTEVQCKGGNVCVQAQDRYYNSS
CSKWACVPCRVRSGGGGSGGGGSGGGGSRVIPVSGPARCLSQSRNLLKTTDDMVKTAREKLKHYSCTAEDIDHED
ITRDQTSTLKTCLPLELHKNESCLATRETSSTTRGSCLPPQKTSLMMTLCLGSIYEDLKMYQTEFQAINAALQNH
NHQQIILDKGMLVAIDELMQSLNHNGETLRQKPPVGEADPYRVKMKLCILLHAFSTRVVTINRVMGYLSSAGGGG
SGGGGSGGGGSEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAIGSVGGPTYYADSV
KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRLAWFDYWGQGTLVTVSSGGSGGEIVLTQSPGTLSLSPGER
ATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQSG
KGPLTFGQGTKVEIKDIGGGSGGGGSGGGGEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGL
EWVSAIGSVGGPTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRLAWFDYWGQGTLVTVSSGGSG
GEIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTL
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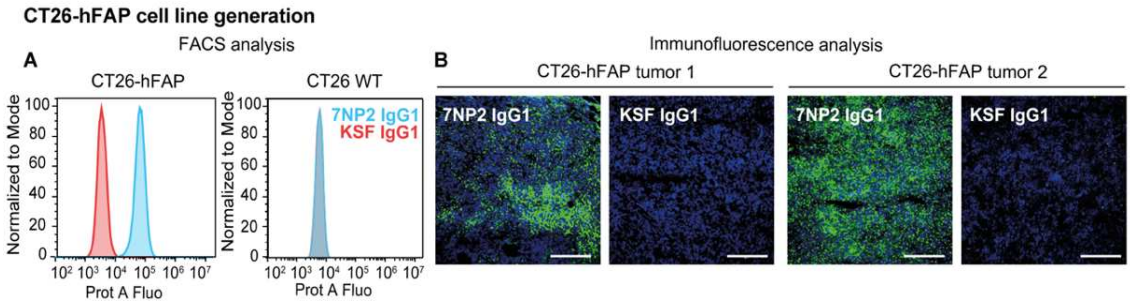


Figure S9. CT26-hFAP stable cell line generation

Development of stable cell lines expressing human FAP was performed as previously described [5-6]. Briefly, CT26 cells were seeded at a density of 1 million cells in a 24 well-plate using RPMI-1640. Next day, 10 μL of the virus aliquot and polybrene (Santa Cruz Biotechnology) to the final concentration of 8 μg/mL were added to the cells. The plate was then centrifuged at 1,000 x g for 90 minutes at 32 °C to enhance the cellular uptake of the virus. In the following day, the media was changed, and cells were kept growing and expanding for three weeks according to the general cell culture procedures. Finally, positive cells were sorted at the flow cytometry facility (BD FACS Aria III) using 7NP2 IgG1-FITC. A. FACS analysis on CT26-hFAP and CT26 WT with 7NP2 IgG1-FITC and KSF IgG1-FITC. B. Immunofluorescence analysis on CT26-hFAP tissue slides from different tumors grown in BALB/c mice. Cryostat sections were stained with ant-FITC (green, AlexaFluor 488) and DAPI (blue). Representative pictures of the samples were taken at 20x magnification, scale bars = 100 µm.

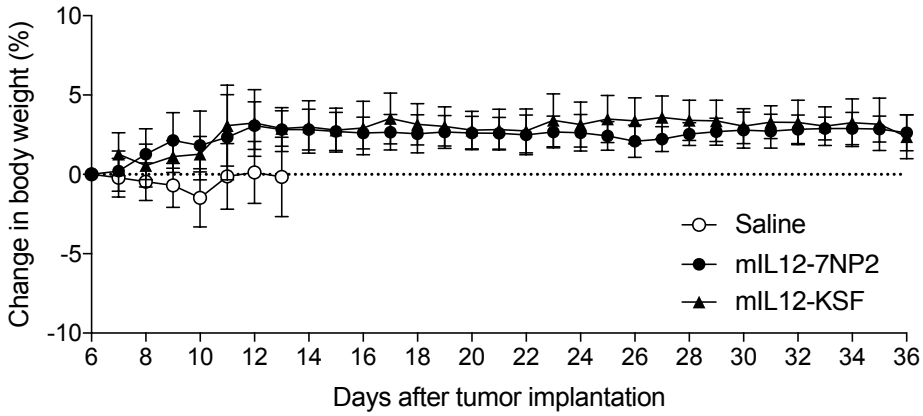


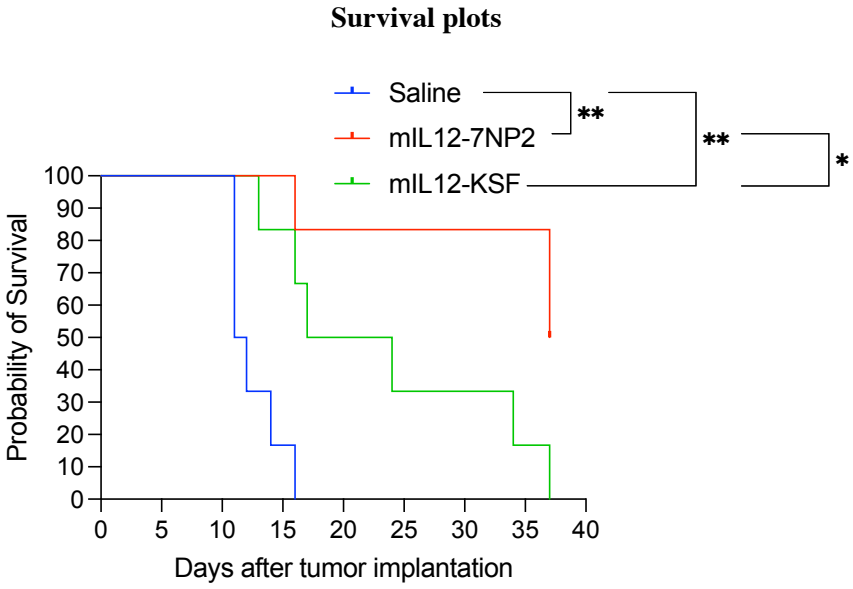
Figure S10. Body weight changes of mice treated with mIL12-7NP2 in SKRC52-hFAP tumor bearing mice.

Body weight changes of mice treated with mIL12-7NP2, mIL12-KSF and saline respectively.

Figure S11. Statistical analysis of SKRC52-hFAP tumor volumes and survival plots.

Tumor volumes SKRC52-hFAP at Day 11

Saline vs mIL12-7NP2	$p < 0.0001$	(****)
Saline vs mIL12-KSF	$p = 0.0054$	(**)
mIL12-7NP2 vs mIL12-KSF	$p = 0.0101$	(*)



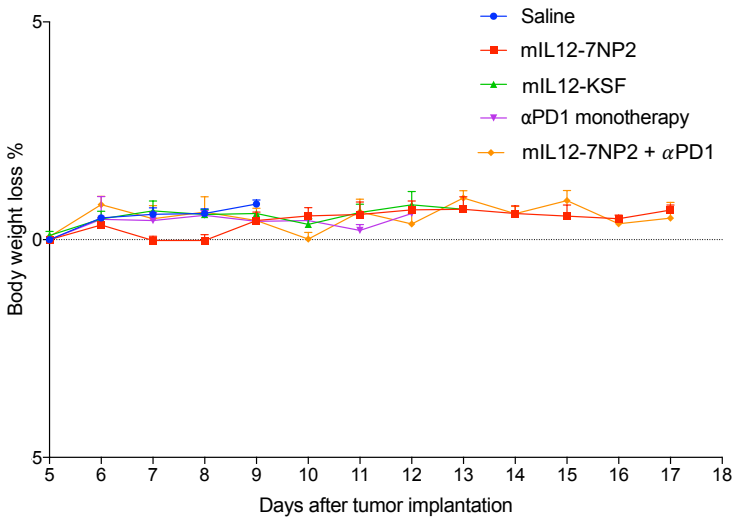


Figure S12. Body weight changes of mice treated with mIL12-7NP2 in combination with aPD1 antibody in CT26-hFAP tumor bearing mice.

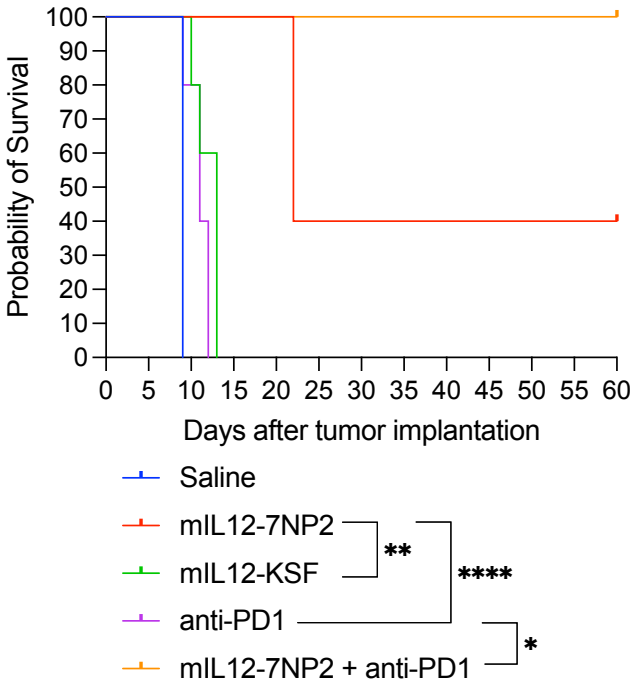
Body weight changes of mice treated with saline, aPD-1 antibody monotherapy, mIL12-KSF (negative control), mIL12-7NP2 monotherapy and mIL12-7NP2 + aPD-1 antibody respectively.

Figure S13. Statistical analysis of CT26-hFAP tumor volumes and survival plots.

Tumor volumes CT26-hFAP at Day 10

mIL12-7NP2 vs mIL12-KSF	p = 0.0379	(*)
mIL12-7NP2 vs anti-PD1	p = 0.0447	(*)
mIL12-7NP2 + anti-PD1 vs mIL12-KSF	p = 0.0363	(*)
mIL12-7NP2 + anti-PD1 vs anti-PD1	p = 0.0446	(*)
mIL12-7NP2 vs mIL12-7NP2 + anti-PD1	p = 0.9950	(ns)
mIL12-KSF vs anti-PD1	p = 0.1531	(ns)

Survival plots



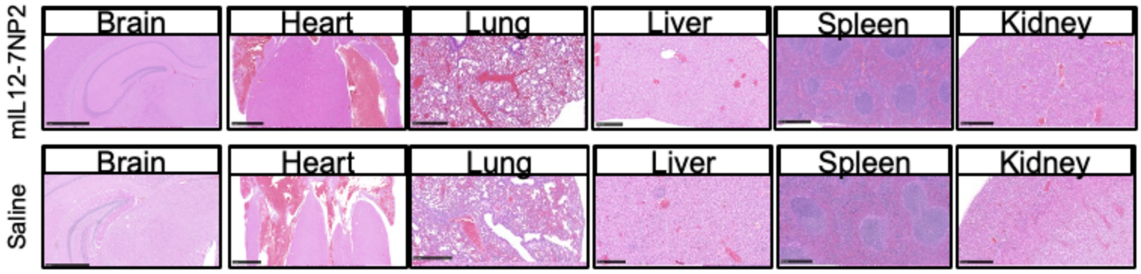


Figure S14. Hematoxylin and eosin staining of organs

Pathological findings of CT26-hFAP tumor bearing mice administered with PBS or mIL12-7NP2. Scale bars: 1mm for brain and heart; 500μm for lung, spleen and kidney; 250μm for liver.

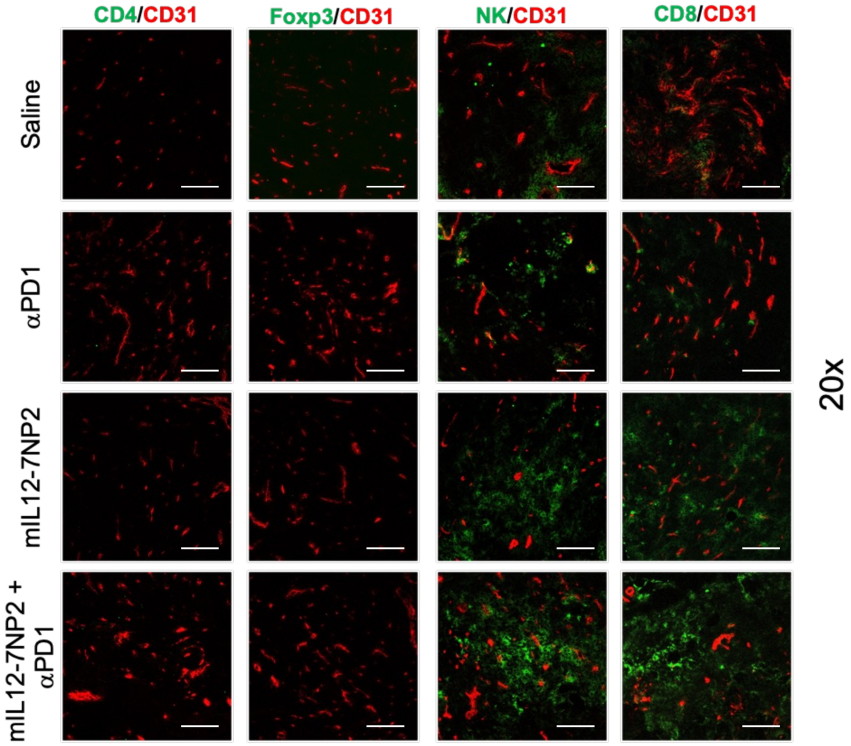


Figure S15. Immunofluorescence analysis of tumor-infiltrating cells on CT26-hFAP tumor sections

Immunofluorescence analysis of tumor-infiltrating cells on CT26-hFAP tumor sections 24 hours after treatment with PBS, anti-PD1, mIL12-7NP2 and the combination mIL12-7NP2+anti-PD1 antibody. marker specific for NK cells (NKp46), CD4+ T cells (CD4), CD8+ T cells (CD8), and Tregs (FoxP3)

were stained in green (Alexa Fluor 488), anti-CD31 for blood vessels (red, Alexa Fluor 594), 20x magnification; scale bars, 100 μm.

Figure S16. Amino acid sequence of IL12-7NP2. Sequence from PCT/EP2022/060776

IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVLSH
SLLLLHKKEDGIWSTDILKDQKEPKNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLS
AERVRGDNKEYEYSVECQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIRDIIKPDPPKNLQLKPLKNSRQVE
VSWEYPDTWSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCSGG
GGSGGGGSGGGGSRNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDITKDKTSTVEACL
PLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVI
DELMQALNFNSETVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNASGGGGSGGGGSGGGGSEVQLLE
SGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAIGSVGGPTYYADSVKGRFTISRDNSKNTLYLQM
NSLRAEDTAVYYCAKRLAWFDYWGQGTLVTVSSGGSGGEIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQ
KPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQSGKGPLTFGQGTKVEIKDIGGGSGG
GGSGGGGEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAIGSVGGPTYYADSVKGRFTI
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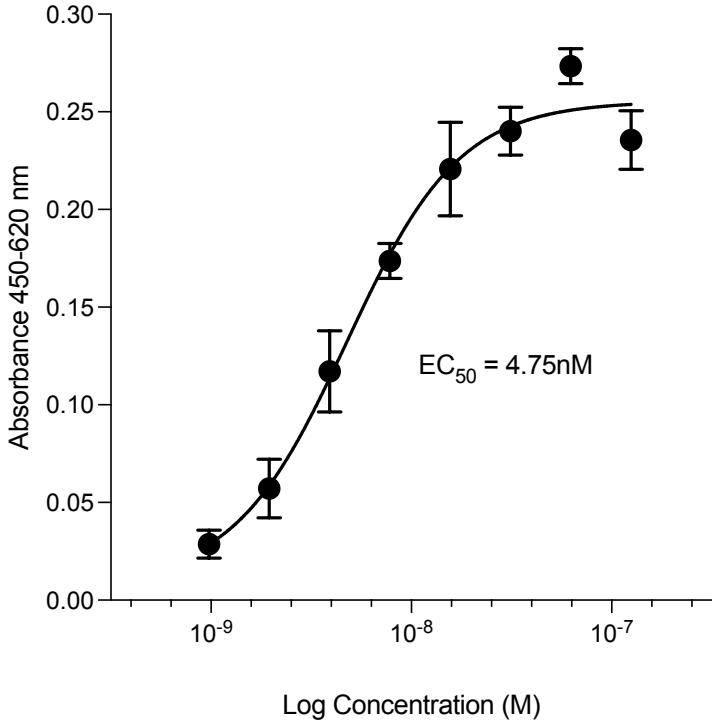
KVEIK

Figure S17. Titration ELISA of 7NP2 scFv on cynomolgus monkey FAP



A serial dilution of 7NP2 scFv was tested on cynomolgus monkey FAP coated-wells and detected with ProteinA-HRP.

Extended Safety Toxicology data

Flow Cytometry Analysis of *Cynomolgus* monkeys peripheral blood cells

The immunophenotyping analysis of peripheral blood cells was performed by a "lyse and wash" direct immunofluorescence staining of the surface markers followed by flow cytometry analysis. The stained samples were kept refrigerated, protected from light, until acquisition to the cytometer on the same day. FACS data acquisition was performed with a Becton Dickinson flow cytometer equipped with 405, 488 and 635 laser lines, BD FACSLyric. BD FACSuite software was used for data collection and analysis. Instrument calibration was performed each time the apparatus was used at start of the working day, as recommended by the manufacturer.

Quantification of IL6, IP10, IFNγ and Neopterin in *Cynomolgus* Monkey serum

Blood collection time points for cytokine plasma levels were at day 1 (pre-dose, 6, 12, 24 and 96 hours after dosing) and at day 22 (pre-dose, 6, 12, 24 and 96 hours after dosing).

Quantitative measurements of IFN-γ and IP10 in monkey serum samples were determined by ELISA methods using commercial kits: Rhesus Macaque IFNγ ELISA kit (Thermo Fisher Cat. Nr. EP8RB) and Monkey IP10 ELISA kit (Thermo Fisher Cat. Nr. EP15RB), respectively. Quantitative determination of *Cynomolgus* Monkey Interleukin 6 in serum, was determined using a homogeneous AlphaLISA assay (no wash steps). Reagents are provided with the commercial kit Perkin Elmer, Cat. Nr. AL223C, while Cynomolgus IL6 standard is purchased separately (Cat. Nr. AL555S). Quantitative measurement of Neopterin in monkey serum samples was determined by competitive ELISA method using commercial kit: Neopterin ELISA kit (IBL International, Cat. Nr. RE59321).

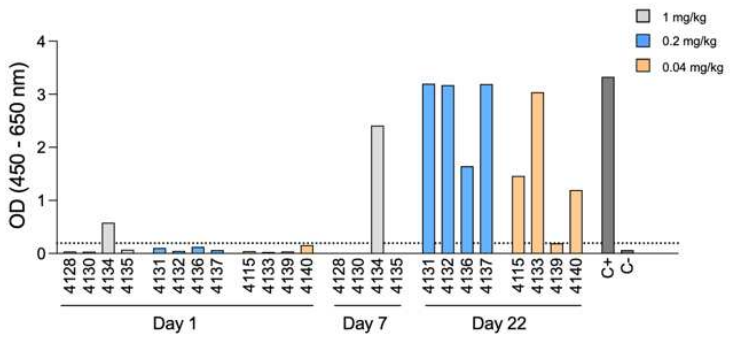


Figure S18. Safety Toxicology studies in *Cynomolgus* monkeys – Anti-drug Antibody

Anti-drug Antibodies (ADA) Parameters Evaluated at pre-dose, day 7 and day 22 plotted for single monkey administered with IL12-7NP2 (1 mg/kg, 0.2 mg/kg and 0.04 mg/kg).