

The targeted delivery of interleukin-12 to the carcinoembryonic antigen increases the intratumoral density of NK and CD8⁺ T cell in an immunocompetent mouse model of colorectal cancer

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Abstract: The recent success achieved by immune checkpoint inhibitors in the field of immuno-oncology has been less evident for the treatment of metastatic colorectal cancer (mCRC) patients. To date, cancer immunotherapy has been efficacious only in few patients bearing high mutational burden (less than 25%) mCRCs. In this Communication, we report the generation of a novel antibody cytokine fusion protein (termed Sm3E-mIL12) targeting the CRC-associated carcinoembryonic antigen (CEA). The antibody moiety bound avidly to CEA when immobilized on solid supports, and selectively stained C51 tumor cells transfected with the antigen (C51-CEA). The cytokine payload retained full activity *in vitro*, as compared to the parental recombinant interleukin-12 (IL12). *Ex vivo* microscopic analyses revealed a homogenous distribution of Sm3E-mIL12 in the neoplastic mass upon intravenous administration. *In vivo*, Sm3E-mIL12 was well tolerated up to 180 µg per mouse. The targeted delivery of IL12 to CEA-expressing C51 carcinomas led to durable complete responses in 60% of the treated mice. The intratumoral density of immune effector cells was markedly increased after the third injection of Sm3E-mIL12, in keeping with the progressive regression of the neoplastic mass. The data suggest that a fully human analogue may be considered for the treatment of patients with mCRC.

Keywords: Antibody-cytokine fusions; interleukin-12; tumor-infiltrating lymphocytes (TILs); carcinoembryonic antigen; colorectal cancer

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Introduction

Colorectal cancer represents the second leading cause of tumor-related deaths in the United States (1,2). Over the last decades, implementation of organized screening programs in the asymptomatic population have substantially decreased CRC incidence and overall mortality, by detecting cancer in its early stage (3,4). Nonetheless, ~22% of patients with CRC are still diagnosed with a metastatic condition (i.e., stage IV), or progress to late stage disease (5,6). Remarkable advances in the development of anti-cancer therapies have spawned multiple therapeutic strategies for the treatment of mCRC. First-line therapies involve the combination of 5-fluorouracil (FU)/leucovorin (LV) and oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) with monoclonal antibodies targeting the vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR) (7). Despite these advances, the 5-year survival rate of patients with mCRC continues to hover below 15% and new therapeutic strategies are therefore urgently needed (8).

In initial clinical studies including unselected groups of CRC patients, immune checkpoint inhibitors exhibited very limited anti-tumor activity. Closer evaluations underlined a small subset of patients experiencing long-term cancer remissions when treated with anti-PD1 antibodies (9). Genetic analyses revealed that these long-term survivors had highly mutated tumors (10,11), characterized by deficient mismatch repair (dMMR) and microsatellite instability-high (MSI-H) (8,12-14). These features are typically associated with cancer immunogenicity, presence of a substantial number of tumor-infiltrating lymphocytes (TILs) (15), and upregulation of immunological checkpoints. On the other hand, immune checkpoint inhibitors are not efficacious against MMR-proficient (pMMR) tumors (16), which account for the majority of the diagnosed mCRC. As these setbacks have been associated with immunologically poor tumors (8), strategies aiming at boosting cell-based immunity may be beneficial for this large proportion of patients with pMMR tumors.

In this study, we explored the antibody-based delivery of IL12 to the tumor microenvironment as a strategy to increase the intratumoral density of effector cells. We generated a novel fusion protein consisting of murine interleukin-12 sequentially fused with a peptidic linker to the Sm3E antibody in tandem diabody format (Sm3E-mIL12). Murine IL12 was used as surrogate therapeutic payload since human IL12 does not cross-react with the cognate murine IL12 receptor. The Sm3E antibody specifically targets the carcinoembryonic antigen (also called CEA or CEACAM5), a validated tumor-associated protein of gastrointestinal carcinomas (17,18). In healthy individuals, CEA's expression is restricted to the apical surface of mature enterocytes (19), making the antigen virtually inaccessible from systemic circulation. However, when these epithelial cells turn malignant, the polarized expression of CEA breaks down, exposing the antigen to the vascular and lymphatic systems (20). As a result, serum levels of soluble CEA are routinely monitored to assess treatment response or disease recurrence in patients with CRC (21,22). Moreover, the selective accessibility in tumors of the membrane-bound CEA, make this protein an ideal target for monoclonal antibody-based therapies (23-25). In this study, we used a clinically translatable CEA-expressing tumor model (C51-CEA) previously established in our group, in order to investigate the anti-cancer potential of Sm3E-mIL12 in an immunocompetent setting (26).

The novel Sm3E-mIL12 immunocytokine retained a high binding affinity to the cognate antigen, and was potently active *in vitro* in terms of IFN-γ stimulation. Furthermore, the fusion protein was able to selectively localize in murine CEA subcutaneous tumors, while sparing healthy organs. Sm3E-mIL12 led to durable cancer eradications in 60% of the treated BALB/c mice, bearing established C51-CEA colon carcinomas. Microscopic analysis of the neoplastic masses revealed that the density of effector TILs was substantially increased after Sm3E-mIL12 treatment. The results of our study provide a rationale for the targeted delivery of IL12 for the treatment of pMMR colorectal cancer, possibly in combination with immune checkpoint inhibitors.

Material and methods

Cell lines, animals and tumor models

CHO-S (Invitrogen; CVCL_7183), and C51 colon carcinoma cells (kindly provided by Dr. M.P. Colombo, Department of Experimental Oncology, Istituto Nazionale Per Lo Studio E La Cura Dei Tumori, Milan, Italy) were expanded and stored as cryopreserved aliquots in liquid nitrogen. Cells were grown according to the manufacturer's protocol and kept in culture for no longer than 14 passages. Authentication of the cell lines including post-freeze stability, growth properties and morphology, test for mycoplasma contamination, isoenzyme assay, and sterility were performed by the cell bank before shipment. C51 cells were stably transfected with CEA as previously described (26). All experiments were performed with mycoplasma-free cells. Seven to eight-week-old female BALB/c mice were obtained from Janvier; 2-4×10⁶ cells (C51 colon carcinoma), were implanted subcutaneously in the left flank of the mice.

Cloning, expression and in vitro protein characterization.

The format chosen for Sm3E-mIL12 was inspired by previous work in our laboratory with F8 and L19 antibody derivatives (27). The sequence of the gene is reported in *Figure S1*. The insert was cloned into NheI/NotI of pcDNA3.1 (+) (Invitrogen), allowing the expression in mammalian cells. Sm3E-mIL12 was expressed using transient gene expression in Chinese Hamster Ovary (CHO) cells, using previously described procedures (28,29). The product was purified from the cell culture medium by affinity chromatography using a protein A affinity column and analyzed by SDS-PAGE, size exclusion chromatography (Superdex200 10/300GL, Healthcare), enzyme-linked immunosorbent assay (ELISA), flow cytometry (2L-Cytoflex, Beckman-Coulter), and surface plasmon resonance analysis (Biacore S200, GE Healthcare)

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on an CEA antigen-coated sensor chip (Sensor Chip SA, GE Healthcare; 10231984), following previously described protocols (26).

For the ELISA assay, soluble CEA was coated onto Nunc MaxiSorpTM wells (ThermoFisher, 44-2404-21). Binding was tested using Sm3E-mIL12, IgG_{2a}(Sm3E) or KSF-mIL12 (KSF is an antibody specific to hen-egg lysozyme, used as negative control), which were subsequently detected with protein L-HRP (ThermoFisher, 32420). Positive binding was eventually confirmed through the reaction of the peroxidase with the TMB-Blotting Substrate Solution. Samples were analysed with a SpectraMax[®] Paradigm multimode detection platform (Molecular Devices) at 450 nm, and results are shown in terms of fold change in absorbance between Sm3E-mIL12 or IgG_{2a}(Sm3E), as compared to the negative control.

Flow cytometry analysis was performed on C51.wt or C51-CEA cell. Proteins were labelled with fluorescein 5-isothiocyanate (FITC, F7250 Sigma) following the manufacturer's protocol. Cells were resuspended at 3 mio/mL in FACS buffer (2% BSA, 2 mM EDTA in PBS) and membrane-bound CEA was detected using FITC-labelled Sm3E-mIL12 or KSF-mIL12 at 1 µg/mL. Data were analysed using a 2L-CytoFlex flow cytometer and subsequently with FlowJo 9 software suite (FlowJo LLC).

Protein stability assay

Sm3E-mIL12 was subjected to a protein stability assay in mouse serum (Sigma, M5905). The protein was incubated for 72 h at room temperature at 40 µg/mL (concentration at t0 upon intravenous injection, considering a dose of 60 µg). Sm3E-mIL12 diluted in PBS (40 µg/mL) was used as positive control, while mouse serum was as negative control for the experiment. Stability was analysed in terms of binding in ELISA, on CEA-coated Nunc MaxiSorpTM wells. The fusion protein was detected with goat anti-murine IL12 p70 (ThermoFisher) and rabbit anti-goat HRP (Dako) antibodies. The results are shown in *Figure S2*.

Bioactivity assay

Sm3E-mIL12, and recombinant mIL12 (BioLegend) were subjected to IFN- γ release assay. Lymphocytes were isolated from freshly dissected tumor-draining lymphnodes of saline treated 129/SvEv mice, bearing F9 teratocarcinoma. After red blood cell lysis, lymphocytes were resuspended at 3×10⁶ cells/mL in RPMI-1640 (Gibco; 21875-034) supplemented with antibiotic-antimycotic (Gibco; 15240-062), 10% Fetal Bovine Serum (Gibco; 10270-106), 50 μ M β -mercaptoethanol (Sigma Aldrich). 100 μ L of the cell suspension was incubated for 4 days at 37 °C and 5% CO₂ with a serial dilution of the IL12 derivatives. IFN- γ levels from cultured supernatants were analysed by enzyme-linked immunosorbent assay (BioLegend; 430804) following the manufacturer's protocol.

Ex vivo biodistribution analysis

The tumor homing ability of Sm3E-mIL12 was assessed by an ex vivo biodistribution study; 200 µg of FITClabelled Sm3E-mIL12 or KSF-mIL12 were injected into the lateral tail vein of BALB/c mice (Janvier) bearing C51-CEA tumors. Mice were sacrificed 24h after the injection. Organs were excised and embedded in cryoembedding medium (ThermoScientific) from which cryostat tissue sections (8-10 µm thickness) were made. FITC signal was amplified using rabbit anti-FITC (Bio-Rad, 4510-7804) and goat anti-rabbit AlexaFluor488 (Invitrogen, A1108). Signal amplification was required for the analysis with the wide field Axioskop2 mot plus microscope (Zeiss). For vascular staining, goat anti-CD31 (R&D System, AF3628) and antigoat AlexaFluor594 (Invitrogen, A11058) antibodies were used. The quantification of tumor and organs uptake of the products, using Image J software, is depicted in Figure S3.

Therapy studies

Mice were monitored daily. Tumor volume was measured using a caliper (volume = length × width² ×0.5). Mice were intravenously injected with 20, 45 and 60 µg of Sm3EmIL12, and 60 µg KSF-mIL12, every second day for three times. The therapeutic agent was diluted in Phosphate Buffer Saline (PBS; Gibco). Animals were euthanized when tumors reached a maximum of 1,500 mm³ (n=5 mice per group).

Analysis of tumor-infiltrating lymphocytes (TILs)

To analyse TILs, mice were injected intravenously three times, every second day, with 60 µg Sm3E-mIL12, 60 µg KSF-mIL12 or saline. Treated mice were euthanised 24 h after the first and third injection. Tumors were excised and embedded in cryoembedding medium (ThermoScientific) and the corresponding cryostat tissue sections (8–10 µm thickness) were stained using the following primary antibodies: goat anti-CD31 (R&D System;



Figure 1 *In vitro* characterization of Sm3E-mIL12. (A) Schematic representation of Sm3E-mIL12. The fusion protein consists of the murine heterodimeric IL12 followed by the Sm3E antibody in tandem diabody format. (B and C) SDS-PAGE in non-reducing (NR) and reducing (R) conditions, and size exclusion chromatography of Sm3E-mIL12. L = Ladder. (D) ELISA performed on CEA-coated wells. Results show fold change in absorbance (450 nm) of Sm3E-mIL12 and IgG_{2a} (Sm3E) compared to the control antibody (KSF-mIL12). (E) Surface Plasmon Resonance analysis of Sm3E-mIL12 at 125, 250 and 500 nM, on CEA-coated sensor chip. (F) Flow cytometry of Sm3E-mIL12 showing selective binding to C51-CEA cells. (G) IFN- γ release assay by Sm3E-mIL12 and recombinant murine IL12 in 129/SvEv mice's tumor-draining lymph nodes.

AF3628), rabbit anti-Foxp3 (Invitrogen; 7000914), rat anti-NKp46 antibody (BioLegend; 137602), rabbit anti-CD4 (Sino Biological; 50134-R001), rabbit anti-CD8 (Sino Biological; 50389-R208). Primary antibodies were detected with donkey anti-rabbit AlexaFluor488 (Invitrogen; A11008) or donkey anti-rat AlexaFluor488 (Invitrogen; A21208) and donkey anti-goat AlexaFluor594 (Invitrogen; A21209) or donkey anti-goat AlexaFluor488 (Invitrogen; A11055). Slides were mounted with fluorescent mounting medium (Dako Agilent) and analyzed with Nikon Eclipse Ti-E fully-integrated, motorized inverted microscope (Tokyo, Japan).

Results

Cloning and characterization of the Sm3E-mIL12 fusion protein

Figure 1A shows a schematic representation of the novel Sm3E-mIL12 immunocytokine. The p40 and p35 subunits of murine IL12 have been sequentially fused to the N'-terminal site of the Sm3E antibody, cloned in tandem diabody format. The product was purified through protein A affinity chromatography, and impurities were analysed by SDS-PAGE (*Figure 1B*) followed by gel filtration chromatography (*Figure 1C*). Sm3E-mIL12 bound

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Figure 2 Biodistribution analysis of Sm3E-mIL12 and KSF-mIL12. *Ex vivo* microscopic analysis performed on tumor and organ sections of mice bearing C51-CEA subcutaneous lesions. Animals were euthanized 24 h after a single injection of Saline (negative control), 200 µg of FITC-labelled Sm3E-mIL12 or 200 µg of FITC-labelled KSF-mIL12.

efficiently to both soluble and membrane-bound CEA (C51-CEA), as evidenced by ELISA, Surface Plasmon Resonance and Flow Cytometry binding studies (*Figure 1D,E,F*). The ability of the fusion protein to induce IFN- γ production was confirmed in a cell-based assay using murine IL12 as reference, yielding with a EC50 values of 1.583 and 1.753 pM, respectively (*Figure 1G*).

Biodistribution study of FITC-labelled Sm3E-mIL12 and KSF-mIL12

The ability of Sm3E-mIL12 to localize at the tumor site was assessed through an *in vivo* biodistribution study in immunocompetent BALB/c mice, bearing C51-CEA lesions (saline treatment was used as negative control). 24 h after intravenous administration, the fusion proteins were detected *ex vivo* by immunofluorescent staining (*Figure 2*). While the two products were undetectable in healthy organs, the tumor uptake was substantially higher when IL12 was fused to the tumor-targeting antibody (i.e., Sm3E-mIL12) as compared to KSF-mIL12. Moreover, as evidenced by a protein stability assay, Sm3E-mIL12 retained binding to the cognate antigen after being incubated for 72 h in mouse serum (*Figure S2*).

Therapy experiments

In a preliminary dose escalation experiment conducted in BALB/c mice bearing C51-CEA subcutaneous tumors, Sm3E-mIL12 was well tolerated up to 60 µg, as evidenced by the absence of body weight loss (*Figure 3A*). Moreover, a cumulative dose of 180 µg exerted a potent anti-cancer cancer effect on small tumors. In a therapy study performed on mice carrying well established subcutaneous lesions, Sm3E-mIL12 exerted a potent tumor-growth inhibition leading to durable complete responses in 60% of the treated animals. By contrast, the untargeted IL12 (i.e., KSF-mIL12) did not show a comparable therapeutic performance (*Figure 3B*).

Microscopic analysis of tumor-infiltrating lymphocytes

In order to gain insights on the mechanism of action of Sm3E-mIL12, we analysed the immune cell infiltrate of C51-CEA tumors, excised 24 h after the first and last injection of the immunocytokine (i.e., 1 and 5 days after the first injection). A progressive infiltration of NK cells, CD4⁺ and CD8⁺ T cells within the tumor mass could be observed along with Sm3E-mIL12 treatment (*Figure 3C*). By contrast, the number of regulatory T cells remained



Figure 3 Therapy study and microscopic analysis of tumor-infiltrating lymphocytes. (A) Dose escalation study of Sm3E-mIL12 in mice bearing C51-CEA lesions intravenously injected every second day (arrows) (B) Therapeutic performance of Saline, 60 µg of KSF-mIL12 and 60 µg of Sm3E-mIL12 in C51-CEA-bearing BALB/c mice, injected every second day (arrows). Treatment started when subcutaneous tumors reached an approximate size of 100 mm³. Statistical differences were assessed between KSF-mIL12 (non-targeted product) and Sm3E-mIL12 (targeted product). ****, P<0.0001 (regular two-way ANOVA test with Bonferroni post-test). Data represent mean tumor volume and body weight change (± SEM). CR, complete response. n=5 mice per group. (C) *ex vivo* immunofluorescence analysis on C51-CEA sections, 24 h after the first and third injection(s) of Saline, 60 µg of KSF-mIL12 or 60 µg of Sm3E-mIL12. Markers specific for apoptotic cells (caspase-3), NK cells (NKp46), Tregs (Foxp3), CD4⁺ T cells (CD4), CD8⁺ T cells (CD8) were used (green). Blood vessels were stained with an anti-CD31 antibody (red). 20× magnification; scale bars =100 µm.

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unchanged.

Discussion

In this Communication, we have described the characterization of a novel immunocytokine consisting of murine interleukin-12 fused to the tumor-targeted Sm3E antibody (Sm3E-mIL12) (Figure 1). Sm3E is a humanized immunoglobulin with high binding affinity to both soluble and cell membrane-bound CEA (Kd =20 pM). Sm3E-mIL12 efficiently localized at the tumor site and not in healthy organs, thus potentially reducing systemic toxicity (Figure 2). Sm3E-mIL12 led to a complete tumor eradication 60% of the treated mice, while murine IL12 fused to KSF (a control antibody specific to hen egg lysozyme) induced only a transient and not significant inhibition of the tumor growth (Figure 3B). The anti-cancer activity of Sm3E-m-IL12 correlated with a progressive increase of CD4⁺, CD8⁺ T cells and NK cells within the tumor mass, consistent with the regression of the neoplastic lesions (Figure 3C). The increase of effector cells might derive from an expansion of pre-existent tumor-resident lymphocytes, or from newly infiltrating leukocytes attracted at the site of disease by chemokine gradients. Previous work of our group showed a dramatic elevation of IFN-inducible protein 10 (IP10) and of monokine-induced by IFNy (MIG) in cancer lysates upon treatment with a tumor-targeted IL12 (30). Furthermore, depletion experiments have shown that the presence of IP10 and MIG is crucial, at least in mouse models, to achieve a robust T cell infiltration within the tumor mass (31).

CEA is a validated tumor-associated antigen discovered by Phil Gold and Samuel Freeman in 1965 (32). In healthy individuals, the expression of CEA is restricted to the apical surface of polarized epithelial cells across the gastrointestinal tract (21). Therefore, the membranebound antigen is not accessible to therapeutic proteins from systemic circulation. However, when the epithelial cells become malignant, CEA can be found around the whole cell surface as a result of tumor cells differentiation (21). The selective accessibility of CEA in cancer patients cancer has made it an ideal target for both imaging and therapeutic applications (24,25).

At present, systemic therapeutic strategies are not efficacious in controlling mCRC, except for a small subset of patients with highly mutated cancers (i.e., dMMR-MSI-H tumors) that respond well to immune checkpoint inhibitors (9). The high response rate to immunotherapy observed in this population has been correlated with the presence of a substantial number of TILs (33). However, the majority of mCRC have a pMMR phenotype (~85%) characterized by low immunogenicity, few TILs, and are consequently irresponsive to immunotherapy. It is now clear that the presence of pre-existent tumor-specific lymphocytes represents a crucial biomarker of responsiveness to anti-PD1 treatments (34-36). Beyond immune checkpoint inhibitors, bispecific antibodies are currently being investigated in pMMR CRC (37). Cibisatamab, a bispecific antibody which bridges CEA⁺ and CD3⁺ cells, showed promising anti-cancer activity in combination with Tecentriq[®] (atezolizumab) (NCT02650713) (38,39). In principle, an immunocytokines directed to a tumor cell membrane antigen may be equivalent to a bispecific entity, creating an immunological synapse between the cancer cell and suitable leukocytes expressing the cognate cytokine receptors (e.g., T and NK cells).

IL12 is a strong modulator of the immune system which boosts the activity of T cells and NK cells. However, recombinant IL12 induces life threatening side-effects at really low doses (i.e., 500 ng/kg in human patients), thus preventing dose escalation to therapeutically effective regimens (40,41). The fusion of IL12 to the tumor-targeting Sm3E antibody provides a strategy to reduce exposure of the pro-inflammatory cytokine to healthy organs, while enhancing activity at the site of disease. In this study, the antibody-based delivery of IL12 to the neoplastic mass has shown to strongly enhance the number of TILs. Taken together, our data suggest that Sm3E-IL12 may be a suitable product to combine with immune checkpoint inhibitors for the treatment of pMMR which lack of effector T cells.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi. org/10.21037/jgo.2020.04.02). The authors have no

conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (license number 04/2018) granted by the Veterinäramt des Kantons Zürich, Switzerland, in compliance with the Swiss Animal Protection Act (TSchG) and the Swiss Animal Protection Ordinance (TSchV).

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MWELEKDVYVVEVDWTPDAPGETVNLTCDTPEEDDITWTSDQRHGVIGSGKTLTITVKEFLDAGQYTCHKGGETLSHSHLLLHKKENGIWSTEIL KNFKNKTFLKCEAPNYSGRFTCSWLVQRNMDLKFNIKSSSSSPDSRAVTCGMASLSAEKVTLDQRDYEKYSVSCQEDVTCPTAEETLPIELALE ARQQNKYENYSTSFFIRDIIKPDPPKNLQMRPLKNSQVEVSWEYPDSWSTPHSYFSLKFFVRIQRKKEKMKETEEGCNQKGAFLVERTSTEVQC KGGNVCVQAQDRYYNSSCSKWACVPCRVRSGGGGSGGGGSGGGGSGGGGSGVIPVSGPARCLSQSRNLLKTTDDMVKTAREKLKHYSCTAEDIDH EDITRDQTSTLKTCLPLELHKNESCLATRETSSTTRGSCLPPQKTSLMMTLCLGSIYEDLKMYQTEFQAINAALQNHNHQQIILDKGMLVAIDELM QSLNHNGETLRQKPPVGEADPYRVKMKLCILLHAFSTRVVTINRVMGYLSSAGSADGQVKLEQSGAEVVKPGASVKLSCKASGFNIKDSYMH WLRQGPGQRLEWIGWIDPENGDTEYAPKFQGKATFTTDTSANTAYLGLSSLRPEDTAVYYCNEGTPTGPYYFDYWGQGTLVTVSSGGSGGEN VLTQSPSSMSVSVGDRVTIACSASSSVPYMHWLQQKPGKSPKLLIYLTSNLASGVPSRFSGSGSGTDYSLTISSVQPEDAATYYCQQRSSYPLT FGGGTKLEIKSSSSGSSSSGQVKLEQSGAEVVKPGASVKLSCKASGFNIKDSYMHWLRQGPGQRLEWIGWIDPENGDTEYAPKFQGK ATFTTDTSANTAYLGLSSLRPEDTAVYYCNEGTPTGPYYFDYWGQGTLVTVSSGGSGGENVLTQSPSSMSVSVGDRVTIACSASSSVPYMHWL QQKPGKSPKLLIYLTSNLASGVPSRFSGSGSGTDYSLTISSVQPEDAATYYCQQRSSYPLTFGGGTKLEIK

Figure S1 Protein sequence of Sm3E-mIL12.



Figure S2 Sm3E-mIL12 stability assay in mouse serum. The protein was incubated for 72 h at room temperature at 40 µg/mL (red). Sm3E-mIL12 diluted in PBS (40 µg/mL) was used as positive control (blue), while mouse serum was used as negative control (green). Stability was analysed in terms of binding in ELISA, on CEA-coated Nunc MaxiSorpTM wells. The fusion protein was detected with goat anti-murine IL12 p70 and rabbit anti-goat HRP antibodies.



Figure S3 Quantification of Sm3E-mIL12 and KSF-mIL12 uptake in the tumor and in normal organs using Image J software. Results are showed in terms of fold change in uptake, as compared the Saline group.