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Potential of PD-L1 blockade with a potency-matched dual cytokine antibody-fusion protein leads to cancer eradication in BALB/c-derived tumors but not in other mouse strains

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Abstract

We have recently described a novel therapeutic antibody product (IL2-F8-TNF^{mut}), featuring the simultaneous fusion of murine IL2 and of a TNF mutant with scFv(F8), an antibody specific to the alternatively-spliced extra domain A of fibronectin (EDA). Here, we report on the *in vivo* characterization of the anti-cancer activity of IL2-F8-TNF^{mut} in four immunocompetent murine models of cancer, CT26, WEHI-164, F9 teratocarcinoma and Lewis Lung Carcinoma (LLC), using the product alone or in combination with a monoclonal antibody specific to murine PD-L1. All four models exhibited a strong expression of EDA-fibronectin, which was confined to vascular structures for F9 tumors, while the other three malignancies exhibited a more stromal pattern of staining. A complete and long-lasting tumor eradication of CT26 and WEHI-164 tumors was observed in BALB/c mice when IL2-F8-TNF^{mut} was used in combination with PD-L1 blockade. The combination treatment led to improved tumor growth inhibition in 129/SvEv mice bearing murine teratocarcinoma or in C57BL/6 mice bearing murine LLC, but those cancer cures were difficult to achieve in those models. A microscopic analysis of tumor sections, obtained 24 hours

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Author contributions

R. De Luca and D. Neri: Conception and design; development of methodology; acquisition, analysis and interpretation of data; writing, review and revision of the manuscript.

D. Neri: Study supervision.

Conflicts of interest:

Dario Neri is co-founder, shareholder and member of the board of Philogen, a company working on antibody therapeutics. The authors declare no additional conflict of interest.

Animal source:

Eight week old female BALB/c mice, 129/SvEv mice and C57BL/6 were obtained from Janvier Labs.

Animal research:

Experiments were performed under a project license (license number 27/2015) granted by the Veterinäramt des Kantons Zürich, Switzerland in compliance with the Swiss Animal Protection Act (TSchG) and the Swiss Animal Protection Ordinance (TSchV).

Cell line authentication:

Authentication of the cell lines also including check of post-freeze viability, growth properties and morphology, test for mycoplasma contamination, isoenzyme assay and sterility test were performed by the cell bank before shipment.

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after pharmacological treatment, revealed that the PD-L1 antibody had homogeneously reached tumor cells *in vivo* and that the combination of PD-L1 blockade with IL2-F8-TNF^{mut} stimulated an influx of NK cells and of T cells into the neoplastic mass. These data indicate that potency-matched dual-cytokine fusion proteins may be ideally suited to potentiate the therapeutic activity of immune check-point inhibitors.

Keywords

Immunotherapy; PD-L1 blockade; immunocytokines; IL2; TNF; EDA domain of fibronectin

Introduction

Immune check-point inhibitors are rapidly changing the clinical management of patients with cancer [1,2]. Ipilimumab (blocking CTLA-4), Nivolumab or Pembrolizumab (blocking PD-1) and Avelumab (Blocking PD-L1) [3–6] have gained marketing authorization for the treatment of different types of malignancies, on the basis of an impressive clinical benefit offered to a subset of patients. Unfortunately, not all cancer types and not all patients respond equally well to immune oncology drugs and many combination strategies are currently being investigated, with the aim to improve therapeutic activity with acceptable toxicity [3–7].

The therapeutic activity of immune check-point inhibitors often correlates with the quantity and quality of lymphocyte infiltrate into the solid tumor mass [2]. On one hand, the nature of tumor rejection antigens presented by the tumor influences the anti-cancer activity of specific cytotoxic T cells [8,9]. A growing body of experimental evidence indicates that both mutational load and HLA class I genotype potentially influence response to immunotherapy in patients [10]. Moreover, various experimental strategies are under development, with the aim to turn “cold” tumors “hot”, by increasing the density of lymphocytes in the neoplastic lesions and by tilting the cytokine balance towards a more inflammatory phenotype [11].

Recombinant cytokines (e.g., IL2, TNF, IFN α) have been used for many years, with the aim to boost the patient’s anticancer activity, with some encouraging results. Treatment with recombinant IL2 mediates a long-term survival for a relatively small proportion of patients with metastatic melanoma and renal cell carcinoma [12]. TNF has received marketing authorization in Europe for the treatment of soft-tissue sarcoma with isolated limb perfusion procedures [13], while recombinant IFN α has been used for decades to treat various types of cancer [14]. However, the clinical use of anti-cancer cytokines is often limited to substantial toxicity (sometimes even at sub-milligram dose levels), preventing escalation to therapeutically active regimens [12–15].

In order to improve the therapeutic index of pro-inflammatory cytokines for oncological applications, the fusion of these immunomodulatory payloads with tumor-targeting monoclonal antibodies has been proposed [16–18]. Both intact immunoglobulins and antibody fragments have been used to generate fusion proteins with cytokines (“immunocytokines”). Some of these products have moved to clinical trials [19], on the basis of promising preclinical results.

Our group has previously described two antibody-cytokine fusion proteins (L19-IL2 and L19-TNF), which are currently being investigated in Phase III clinical trials [EudraCT number 2015-002549-72], after having shown encouraging activity in Phase II clinical studies [20–22]. These products recognize the alternatively-spliced extra domain B of fibronectin (EDB), a marker of tumor angiogenesis [23].

We have recently observed that the *simultaneous* delivery of two cytokine payloads to the tumor environment may exhibit a synergistic anticancer effect. For example, the combination of IL2- and TNF-based immunocytokine products was able to eradicate lesions in immunocompetent mouse models [24] and to induce complete responses in patients with stage IIIB/C melanoma [22]. In an attempt to combine the therapeutic activity of IL2 and of TNF into a single molecular entity, we have recently described a novel class of biopharmaceuticals, termed “potency-matched dual cytokine fusions” [25]. The possibility of having both IL2 and TNF moieties incorporated into a single polypeptide would be attractive from a pharmaceutical perspective, as the two synergistic payloads would require the development of only one antibody product. However, we needed to solve a technical challenge, considering the fact that the recommended dose of TNF is approximately ten times lower compared to the one of IL2 [20,26,27]. In order to solve this problem, we have generated a novel fusion protein (termed IL2-F8-TNF^{mut}), featuring the scFv(F8) antibody fragment (specific to the alternatively-spliced EDA domain of fibronectin) fused to murine interleukin-2 (IL2) and to a single-aminoacid TNF mutant [25]. The R111W mutation in the TNF moiety was introduced in order to match the potency of the IL2 and TNF payloads [25]. The EDA domain of fibronectin is expressed in the majority of tumors both in mouse and in man, while being virtually undetectable in normal adult tissues [28]. The F8 antibody recognizes murine and human EDA with identical affinity [29].

In this article, we describe the anti-cancer properties of IL2-F8-TNF^{mut} in combination with an anti-mouse PD-L1 antibody, previously reported to display some level of therapeutic activity in immunocompetent models of cancer [11]. Combination treatment induced cancer cures in BALB/c mice bearing CT26 and WEHI-164 tumors. The same therapeutic modality potentiated immune check-point inhibition and anti-cancer activity in 129/SvEv mice bearing murine F9 teratocarcinoma or in C57BL/6 mice bearing murine LLC, but complete tumor eradications were difficult to achieve in those models. A microscopic investigation of the nature of the immune cell infiltrate following combination treatment provided insights on the mechanism of action.

Materials and Methods

Tumor cell lines and reagents

WEHI-164 fibrosarcoma cells, F9 teratocarcinoma cells, CT26 colon carcinoma cells and LLC Lewis lung carcinoma cells were obtained from the American Type Culture Collection (ATCC) between 2015 and 2017, expanded and stored as cryopreserved aliquots in liquid nitrogen. Cells were grown according to the supplier’s protocol and kept in culture for no longer than 14 passages. The production and purification of IL2-F8-TNF^{mut} was performed as described before [25]. The commercial anti-PD-L1 was purchased at BioXCell (clone 10F.9G2; BE0101).

Tumor models and therapy studies

Tumor cells were implanted subcutaneously in the flank of BALB/c mice using 4×10^6 cells (CT26), 5×10^6 cells (WEHI-164), 15×10^6 cells (F9) in 129/ScEv mice and 2×10^6 cells (LLC) in C57BL/6 mice.

Mice were monitored daily and tumor volume was measured with a calliper (volume = length $\times$ width² $\times$ 0.5). When tumors reached a suitable volume (approx. 70-80 mm³), mice were injected three times into the lateral tail vein with the pharmacological agents. IL2-F8-TNF^{mut} and the commercial anti-PD-L1 antibody (clone 10F.9G2, BioXCell) were dissolved in PBS, also used as negative control, and administered every 48 hours.

IL2-F8-TNF^{mut} was administered at 50 μ g, 20 μ g, 40 μ g and 40 μ g for the therapy in CT26, WEHI-164, F9 and LLC, respectively. The commercial anti-PD-L1 antibody was administered at 200 μ g. In a combination group IL2-F8-TNF^{mut} was administered 6 hours before anti-PD-L1, while in a second combination group anti-PD-L1 was administered 6 hours before IL2-F8-TNF^{mut}.

For the tumor re-challenge study, mice with complete responses were injected subcutaneously with 4×10^6 CT26 cells in the flank.

Immunofluorescence studies

EDA expression was assessed on ice-cold acetone fixed 8- μ m cryostat sections of WEHI-164, CT26, F9 and LLC stained with IL2-F8-TNF^{mut} (final concentration 5 μ g/mL), as negative control IL2-KSF-TNF^{mut} (specific for an irrelevant antigen) was used. Both antibodies were detected with rat anti-IL2 (eBioscience 14-7022-85) and anti-rat AlexaFluor488 (Invitrogen A21208). For vascular staining goat anti-CD31 (R&D AF3628) and anti-goat AlexaFluor594 (Invitrogen A11058) antibodies were used.

For ex-vivo immunofluorescence analysis, mice bearing CT26 or LLC lesions were injected once with IL2-F8-TNF^{mut} + anti-PD-L1 or saline according to the therapy schedule and sacrificed 24h after injection. Tumors were excised and embedded in cryoembedding medium (Thermo Scientific) and cryostat sections (8 μ m) were stained using the following antibodies: rabbit anti-CD4 (SinoBiological 50134-R001), rabbit anti-CD8 (SinoBiological 50389-R208), rabbit anti-FoxP3 (Invitrogen 700914), rabbit anti-Natural Cytotoxicity Receptor 1 (NCR1) (Abcam ab214468), goat anti-CD31 (R&D AF3628) and detected with anti-rat AlexaFluor488 (Invitrogen A21208), anti-rabbit AlexaFluor488 (Invitrogen A11008), anti-goat AlexaFluor594 (Invitrogen A11058). Slides were mounted with fluorescent mounting medium and analysed with Axioskop2 mot plus microscope (Zeiss).

MHC binding prediction of AH1 peptide

AH1 peptide was subjected to MHC class I binding prediction analysis using NetMHCpan 4.0 net [30]. The peptide was assigned the minimal rank for the three BALB/C H-2 alleles D^d, K^d, and L^d and for the two C57BL/6 H-2 alleles D^b and K^b. Weak binding was annotated if the rank calculated by NetMHCpan was below 2% and strong binding was annotated if the rank calculated by NetMHCpan was below 0.5%.

Identification of AH1 from MHC class I complexes of CT26 cells

The purification of MHC class I molecules, mass spectrometric and bioinformatics analysis was essentially performed as described [31]. MHC class I complexes were purified from 10^8 CT26 cells. Eluted peptides were analyzed on a Q Exactive mass spectrometer and data was processed with MaxQuant. After MaxQuant analysis, peptide-to-spectrum-matches of AH1 peptide within 1% false discovery rate were displayed with the MaxQuant Viewer using standard parameters and exported as pdf.

Results

Therapy experiments in immunocompetent mouse models of cancer

Fig. 1 describes the reagents used for therapy experiments and provides information about the murine tumor models used in the study. IL2-F8-TNF^{mut}, assembled into a stable non-covalent homotrimeric structure [Fig. 1a], was produced and purified as previously described [25], while a commercial antibody specific to murine PD-L1 was used as a surrogate for clinically-approved anti-PD-L1 products [11]. The IL2-F8-TNF^{mut} product strongly stained the neovasculature of F9 teratocarcinomas [Fig. 1b], while the three other models used in our study (CT26, WEHI-164 and LLC) displayed a more diffuse stromal staining pattern. When IL2-KSF-TNF^{mut} (a fusion protein with identical format but specific to hen egg lysozyme) was used as negative control in immunofluorescence procedures, no detectable staining was observed in addition to the CD31 signal.

The therapeutic activity of IL2-F8-TNF^{mut} (administered at a dose of $50\mu\text{g}$) was compared with the one of an anti-mouse PD-L1 antibody (used at $200\mu\text{g}$) in immunocompetent BALB/c mice bearing CT26 carcinomas [Fig. 2a]. Treatment with the anti-PD-L1 antibody resulted in a tumor growth profile similar to the one obtained in the saline control group (PBS). A tumor growth inhibition was observed in the IL2-F8-TNF^{mut} group, as well as in the combination group where the anti-PD-L1 antibody had been administered six hours before IL2-F8-TNF^{mut}. By contrast, the combination IL2-F8-TNF^{mut} followed by anti-PD-L1 six hours later induced complete responses in 80% of the treated mice. Animals which had been cured by the combined administration of IL2-F8-TNF^{mut} + anti-PD-L1 were re-challenged with CT26 cells and were found to have acquired a protective immunity against a second tumor implantation [Fig. 2b].

In a second therapy experiment, immunocompetent mice bearing WEHI-164 sarcomas were treated [Fig. 2c]. In this model, which is very sensitive to the action of TNF, the dose of IL2-F8-TNF^{mut} was reduced to $20\mu\text{g}$, in order to assess whether a synergistic effect with anti-PD-L1 treatment could be observed [25]. An extremely potent anti-tumor activity was observed for IL2-F8-TNF^{mut} used as single agent and its combination with PD-L1 blockade led to cancer cures in 4/5 mice, irrespective of the order of administration of the two biopharmaceuticals.

A potent inhibition of tumor cell growth was observed in mice bearing F9 and LLC lesions [Fig. 2d and 2e], but complete cancer eradication was difficult to achieve in these models. All regimens were tolerated as evidenced by the comparison of body weight profiles [Supplementary figure 1].

Microscopic analysis of immune cell infiltrate

The tumor-targeting properties of the commercial anti-PD-L1 antibody were investigated by analyzing sections of CT26 (a tumor that could be cured) or LLC (a tumor that could not be cured in our experimental setting) 24 hours after intravenous administration of 200 μ g of this product. An *ex vivo* immunofluorescence staining, performed using an anti-rat secondary antibody reagent, revealed a patchy uptake of the anti-PD-L1 antibody at the tumor site in both models [Fig. 3a].

A microscopic analysis of leukocyte infiltrate in sections of CT26 tumors [Fig. 3b], obtained 24 hours after a single injection of IL2-F8-TNF^{mut} + anti-PD-L1 or saline, revealed a substantial increase in the intratumoral density of NK cells and CD4+ T cells after combination therapy. Tumor-resident CD8+ T cells were detectable also prior to treatment. By contrast, the density of FoxP3-positive lymphocytes decreased, as a result of pharmacological intervention, indicating product activity against regulatory T cells.

A similar analysis, performed in sections of LLC tumors [Fig. 3c], revealed a distinct increase in the intratumoral density of NK cells, CD4+ T cells and CD8+ T cells after combination therapy. Only a small reduction in the density of FoxP3-positive lymphocytes was observed.

Discussion

The targeted delivery of cytokines to the tumor environment increases the therapeutic index of those immune modulators [32,33,19,34–40] and various antibody-cytokine fusions are currently being investigated in clinical trials for oncological applications. As the combination of cytokines is often required in order to control immunological processes [41], it is attractive to use mixtures of antibody-cytokine fusions in therapy experiments [42–44,24,45] or to develop antibody fusions, featuring multiple cytokine payloads [44,46,25]. Here, we studied the therapeutic activity of a novel antibody-cytokine fusion protein (IL2-F8-TNF^{mut}) in four immunocompetent mouse models of cancer (CT26, WEHI-164, F9 and LLC). The product was used alone or in combination with an anti-PD-L1 antibody (a surrogate for the human-specific PD-L1 blockers Avelumab, Durvalumab and Atezolizumab, which have gained marketing authorization for cancer therapy) [6,47,48].

A potent tumor growth inhibition was observed for IL2-F8-TNF^{mut} used as single agents in CT26 tumors, while PD-L1 blockade had a minimal anti-cancer activity in all experimental models, in keeping with previous reports [11]. The combination of the two products led to long-lasting complete responses, when the immune check-point inhibitor was given after immunocytokine treatment. Pre-administration of IL2-F8-TNF^{mut} may increase vascular permeability of tumor blood vessels, promoting a higher uptake of anti-PD-L1 in the neoplastic lesion. Similar effects in biodistribution studies following pre-administration of TNF fusion proteins have previously been reported by our group [46], the group of Jean-Pierre Mach [49] and the group of Alan Epstein [50]. Analogous findings have also been reported for IL2-based therapeutics [51]. High concentrations of TNF may promote hemorrhagic necrosis [25] and, in certain cases, intravascular blood coagulation [52]. This observation and studies of the immune infiltrate into the tumor mass suggests that the

targeted delivery of pro-inflammatory cytokines (IL2 and TNF) increase the density and activity of lymphocytes in the neoplastic mass, whose activity can be further modulated by PD-L1 blockade.

A potentiation of the therapeutic activity of IL2-F8-TNF^{mut} in combination with the anti-PD-L1 antibody was observed in WEHI-164, leading to cures in most treated animals. Sarcomas are extremely sensitive to the targeted delivery of TNF, which rapidly kills the majority of tumor cells. The anti-cancer role of lymphocytes is mainly confined to the eradication of the few tumor cells, which survive the induction of hemorrhagic necrosis into the neoplastic mass [31,25].

A potent tumor growth inhibition was observed when F9 tumors were treated with IL2-F8-TNF^{mut}, alone or in combination with anti-PD-L1. However, only one mouse could be cured in the combination group. For LLC tumors, no cures were observed and the potency-matched immunocytokine product could mediate only a partial tumor growth inhibition. No apparent benefit was observed in the combination therapy group. No obvious correlation between target antigen expression and therapeutic outcome was observed in these therapy settings, as all models were strongly positive for EDA.

The acquired protective immunity in BALB/c derived tumors indicates a possible role for the AH1 peptide (a peptide derived from the gp70 envelope protein of the murine leukemia virus, endogenous in the BALB/c genome) as a dominant tumor-rejection antigen [31]. The anticancer activity of IL2-F8-TNF^{mut} is mainly driven by CD4+ and CD8+ T cells [25], supporting the notion that the presentation of a suitable tumor-associated antigen on MHC molecules contributes to therapeutic outcome.

At this moment in time, we do not know whether the lower anti-cancer activity observed in the LLC model, strongly positive for EDA(+)-fibronectin, is due to the higher concentration of immunosuppressive mediators or to the absence of suitable immunogenic peptides to support immune surveillance. It has previously been reported that LLC tumors display an anti-inflammatory environment and that they do not respond well to immune check-point inhibition [11]. The observation that the dense infiltration of leukocytes at the tumor site, promoted by pharmacological intervention, does not result in a complete response, correlates with the persistence of regulatory T cells within the tumor mass [Fig. 3c].

A series of publications has shown that LLC and CT26 expresses MHC-I on the cell surface and that specific T-cells (directed against the tumor) recognize putative rejection antigens [31,53–58]. Moreover, the different mouse strain used for LLC tumors (C57BL/6) and for WEHI-164 and CT26 tumors (BALB/c) correlates with a difference in MHC class I alleles [59]. Interestingly, the immunodominant AH1 peptide can be efficiently displayed on H-2L^d of BALB/c tumors, while the same sequence cannot be displayed on H-2D^b or H-2K^b of C57BL/6 tumors. Efficient presentation of AH1 on MHC class I molecules in CT26 cancer cells could be predicted using software tools and was experimentally confirmed by mass-spectrometry-based MHC peptidome analysis [Supplementary table 1 and Supplementary figure 2]. This observation also correlates with the finding that gp70 is strongly expressed in many tumors of BALB/c origin and also in B16F10 melanoma of C57BL/6 origin, while a

very weak expression was seen for LLC, using quantitative PCR analysis [31]. Rejection of LLC tumors and induction of protective immunity has been claimed in the literature [44,60–64]. It might be possible that LLC cells present a different rejection antigen, perhaps derived from gp70, but with a different sequence from the AH1 peptide. The seminal work of Thierry Boon and collaborators [61] featured the treatment of LLC with mutagenic agents. This procedure is likely to increase the immunogenicity of the tumor, as highlighted by Goodenow and collaborators [60]. Virus-mediated transfection of LLC with cytokine genes (e.g., IL2, IL6, IL12) has led to the complete eradication of tumor cells and to the induction of protective immunity [62–64]. These findings can be mimicked by the intratumoral injection of a cocktail of antibody-cytokine fusions [65]. Overall, LLC remains one of the most difficult experimental tumors to treat and cure, using systemic pharmacological approaches.

It is becoming increasingly clear that the ability of tumor cells to efficiently present tumor rejection antigen may represent a dominant feature, that discriminates between tumors that respond (or do not respond) to immunotherapy [10].

IL2-F8-TNF^{mut} is a biopharmaceutical with excellent biodistribution properties in tumor-bearing mice [25]. The product recognizes the alternatively-spliced EDA domain of fibronectin, a marker of tumor angiogenesis. The F8 antibody binds to EDA with identical affinity in mouse and man, thus facilitating clinical translation. EDA is strongly expressed in a large variety of human malignancies, including several types of lymphomas [66], renal cell carcinomas [67], acute leukemias [68], melanoma [69], head&neck tumors [70], as well as high-grade astrocytomas, lung and liver metastases, colon carcinomas, rhabdomyosarcomas and other tumor types [28]. The product may be used alone or, preferably, in combination with immune check-point inhibitors, which are rapidly gaining acceptance as broad-spectrum agents for many cancer indications [1,2].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EDA	Alternatively-spliced Extra Domain A of fibronectin
EDB	Alternatively-spliced Extra Domain B of fibronectin
gp70	Envelope protein of the murine leukemia virus

LLC	Lewis Lung Carcinoma
NCR1	Natural Cytotoxicity receptor 1

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Précis

A potency-matched dual cytokine antibody fusion protein, used alone or in combination with immune check-point inhibitors, induced cancer cures in the majority mice bearing either WEHI-164 or CT26 tumors, but not in mice bearing LLC or F9 lesions.

Fig. 2.

Therapeutic performance of IL2-F8-TNF^{mut} in combination with anti-PD-L1 treatment.

Data represent mean tumor volume $\pm$ SEM. For all therapies mice were injected three times intravenously (black arrows) every 48 hours with either PBS, IL2-F8-TNF^{mut}, 200 μ g anti-mouse PD-L1 or a combination of the two (IL2-F8-TNF^{mut} six hours before anti-PD-L1 or the opposite). n = 5 mice per group (unless stated elsewhere), CR = complete response. (a) Therapy in Balb/c mice bearing CT26 colon carcinoma lesions. Treatment started when tumors reached a volume of 80 mm³, IL2-F8-TNF^{mut} was dosed at 50 μ g. n = 5 mice per

group, CR = complete response. **(b)** Tumor re-challenge study. After 37 days, mice with complete responses were injected subcutaneously with 4×10^6 CT26 cells in the flank. $n = 5$ for PBS group ($n = 4$ from day 12), $n = 4$ for re-challenge group. **(c)** Therapy in Balb/c mice bearing WEHI-164 tumors. Treatment started when tumors reached a volume of 70 mm^3 , IL2-F8-TNF^{mut} was dosed at $20 \mu\text{g}$. **(d)** Therapy in 129/SvEv mice bearing F9 teratocarcinomas. Treatment started when tumors reached a volume of 80 mm^3 , IL2-F8-TNF^{mut} was dosed at $40 \mu\text{g}$. **(e)** Therapy in C57BL/6 mice bearing LLC tumors. Treatment started when tumors reached a volume of 70 mm^3 , IL2-F8-TNF^{mut} was dosed at $40 \mu\text{g}$. $n = 4$ from day 15 for groups IL2-F8-TNF^{mut} + anti-PD-L1 and PBS, from day 16 for group anti-PD-L1 and from day 20 for groups anti-PD-L1 + IL2-F8-TNF^{mut} and IL2-F8-TNF^{mut}.

Fig. 3.

Microscopic analysis of therapeutic performance of IL2-F8-TNF^{mut} in combination with anti-PD-L1 treatment. **(a)** Immunofluorescence analysis of tumor targeting properties of an anti-PD-L1 antibody 24 hours after IL2-F8-TNF^{mut} + anti-PD-L1 treatment in mice bearing CT26 or LLC lesions, cryosections were stained with anti-rat IgG (green, Alexa Fluor 488) and anti-CD31 (red, Alexa Fluor 594), 20x magnification, scale bars = 100 μ m.

Immunofluorescence analysis of tumor infiltrating cells on CT26 **(b)** or LLC **(c)** tumor sections 24 hours after treatment with PBS or IL2-F8-TNF^{mut} + anti-PD-L1, marker specific

for NK cells (NCR1), CD4+ T cells (CD4), CD8+ T cells (CD8) and T regs (FoxP3) were stained in green (Alexa Fluor 488), anti-CD31 (red, Alexa Fluor 594), 20x magnification, scale bars = 100 μ m.