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Running title: TNFα overexpression modulates tumorigenic properties of CRC

Overexpression of TNFα in colorectal cancer cell lines affects tumorigenicity, differentiation, and immune cell infiltration

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The progression of cancer strongly depends on the tumor microenvironment and immune surveillance. Tumor necrosis factor alpha (TNFα), a key inflammatory cytokine, can drive both tumor elimination and promotion, depending on its dose and the type of cancer. Colorectal cancer cell lines HCT 116, HT-29, and melanoma cells A375 engineered to stably overexpress the human TNFα gene were used to induce experimental subcutaneous tumors in two immunodeficient mouse strains: athymic Balb/c-nu/nu and SCID/bg mice. In athymic mice, TNFα-overexpressing cells completely lost their tumorigenicity. In SCID/bg mice, with no mature T and B cells and defective NK cells, the TNFα overexpressing cells formed rudimentary flat ulcerous xenografts with rapidly reduced size, with tumor penetrance of 50-85%. Histopathological analysis revealed necrotic lesions, a more differentiated phenotype of tumor cells forming pseudoglandular structures, and more abundant stromal cells. Intratumoral infiltration of immune cells increased in TNFα-secreting tumors. Positivity of cytokeratins 7 and 20 in colorectal cancer xenografts was decreased. Paradoxically, the expression of ALDH1A1 and ALDH1A3 isoforms, which are important for disease prognosis, was increased. Our study suggests that careful modulation of the tumor microenvironment to a tumor-suppressive one using cytokine TNFα and controlled stimulation of antitumor immunity can contribute to the improvement of cancer treatment.

Key words: TNFα; tumorigenicity; differentiation; tumor-infiltrating lymphocytes; ALDH1; colorectal carcinoma; melanoma

The development and progression of cancer are strongly influenced by the tumor microenvironment (TME) and immune surveillance mechanisms. Tumor necrosis factor α (TNFα), a key pro-inflammatory cytokine, exerts context-dependent effects, contributing to either tumor suppression or promotion depending on its concentration and the specific cancer type [1-3]. The TNFα induces apoptosis of endothelial cells and damage of the endothelium, so the tumor receives fewer nutrients and oxygen, resulting in necrosis. The TNFα/TNFR1-receptor signaling in endothelial cells and pericytes targets tumor angiogenesis and vasculature [4, 5] and probably enhances the synergic

effect of TNFα and chemotherapeutic agents, such as doxorubicin and melphalan in cancer patients [6, 7]. On the other hand, TNFα in low doses can help "normalize" tumor vasculature and vessel perfusion, leading to more effective treatment and drug uptake, and reducing hypoxic areas in the experimental tumor bulk [8]. Tumor cells genetically engineered to overexpress TNFα have significantly affected their tumorigenicity *in vivo*. Their engraftment is blocked after subcutaneous administration (*s.c.*), resulting in smaller tumors with tumor take rate reduced to 50-60%. The growth of lung colonies after intravenous administration is inhibited. Impaired tumorigenicity of engineered TNFα cells was observed in immunocompetent [5, 9] and also in immunodeficient mice [10-12]. Achieved *in vivo* doses of TNFα were extremely low, but very efficient in the inhibition of tumor growth [10]. The effect of TNFα in TME is pleiotropic, modulating the tumor microenvironment via paracrine mechanisms in the context of the cancer tissue. In such markedly reduced xenografts, a 'tumor resistant' or 'tumor suppressive microenvironment' is created [13].

Tumor suppression caused by TNFα overexpression depends on the activation of cells of the immune system (T cells, B cells, NK cells, macrophages, and dendritic cells) using the Stat1-IFN-γ pathway. Inflammatory cell (CD8+, Th1 T, NK cells, APC, M1 macrophages) infiltration in tumor tissue samples has a significant tumor-suppressing effect and is linked with a favorable prognosis. TNFα recruits these tumor-suppressing immune cells [2, 14]. Meta-analyses of cancer risk in patients with chronic, auto-immune inflammatory diseases, such as rheumatoid arthritis, treated with TNFα inhibitors, showed a greater incidence of cancer, or in some studies no increase in the overall risk [15]. Acute inflammatory response, on the other hand, may have a beneficial effect in fighting cancer disease in patients and cause spontaneous regression of tumors, in contrast to chronic inflammation promoting tumor growth [3, 16-18]. In the absence of TNFα *in vivo*, in the TNFα- deficient mice, there is impaired anti-tumor immune surveillance, affected T cell priming, and recruitment of activated lymphocytes to the tumor [19].

Aldehyde dehydrogenase 1 is linked with advanced cancer and poor prognosis, especially its isoforms ALDH1A1 and ALDH1A3 [20]. Increased expression of ALDH1A3 is accompanied by increased metastatic and migratory potential and chemoresistance [21].

The tumor microenvironment includes an extracellular matrix (ECM) consisting of collagens, proteoglycans, and glycoproteins. Its mechano-physical properties, stiffness, and architecture affect tumor progression and treatment efficiency. Desmoplastic tumors with overproduced ECM represent a barrier for immune cells, which is linked with aggressive and treatment-resistant cancers [22]. TNFα mediates ECM remodeling and activity of matrix metalloproteinases. For example, *in vitro* TNFα binds to fibronectin in ECM, attracts monocytes, and triggers their activation into

MMP9-secreting cells [23]. *In vivo* in experimental tumors, TNFα targets ECM through CSG ligand, causing robust immune cell infiltration and reduction of tumor stiffness [24].

In our study, we engineered two lines of colorectal carcinoma cells, HCT 116 and HT-29, and a malignant melanoma cell line A375, to overexpress human TNFα. We employed two different immunodeficient mice strains (athymic and SCID/bg, Table 1) with different residual immune cells, which showed us which (key) immune cells are directly involved in creating a tumor-resistant microenvironment under the TNFα influence and which immune cells are probably responsible for loss of tumorigenicity of TNFα-overexpressing malignant cells *in vivo*. We also evaluated the histopathological and molecular biological characteristics of such tumor-suppressive microenvironment.

Materials and methods

Cell lines and chemicals. Human colorectal carcinoma cell lines HT-29 (#ECACC 91072201); HCT 116 (ATCC® CCL-247™), human melanoma cell line A375 (#ECACC 88113005) were maintained in high-glucose (4.5 mg/ml) Dulbecco's modified Eagle's medium (DMEM) (Biochrom AG, Germany) supplemented with 5 or 10% fetal bovine serum (FBS) (Biochrom AG) and 2 mM glutamine. All cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2. If not stated otherwise, all chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

Retroviral transduction. Proliferating target tumor cells were transduced with Moloney Murine Leukemia virus-derived replication-deficient retroviral particles ST40hTNFα bearing human TNFα transgene as described previously [25]. A multiplicity of infection (MOI) of 5-10 and a transduction efficiency of 50-90% was achieved. Transduction with ST40hTNFα retrovirus was verified by PCR and RT-qPCR. Engineered cell lines were named as HT-29hTNFa, HCT116hTNFa, and A375hTNFa.

***In vivo* experiments.** Six to eight-week-old athymic mice (Balb/c-nu/nu) and SCID/bg mice (Charles River, Germany) (Table 1) were used following the institutional guidelines under the approved protocols. The project was approved by the Institutional Ethic Committee and by the national competence authority (State Veterinary and Food Administration of the Slovak Republic), Registration No. Ro 746/18-221/3 in compliance with Directive 2010/63/EU and Regulation 377/2012 on the protection of animals used for scientific purposes. It was performed in the approved animal facility (license No. SK UCH 02017). Xenografts were induced with a total of 5×10^5 human A375 cells and 2.5×10^5 HT-29 or HCT 116 cells administered subcutaneously into the flanks. Six to eight animals were in the groups. Growing tumors were measured with a caliper, and the tumor volume was calculated using the formula $V=0{,}5236 \times ((\text{width} + \text{length})/2)^3$. Animals with

parental tumor cells were sacrificed on days 27-29. Animals with hTNFα overexpressing cells were sacrificed on day 59 or when aggravation of health status was noticed. Xenograft tissues were analyzed by histology and immunohistochemistry, and/or total RNA for RT-qPCR was isolated from the tissues.

Histopathological and histochemical evaluation. Tissue samples were fixed in 4% neutral buffered formalin solution (Sigma, Aldrich) for 24 h, and then the samples were standardly processed and embedded into paraffin blocks. Blocks were cut on a Hyrax M40 rotary microtome (Zeiss, Germany), and tissue sections were placed on Star Frost® glass slides (Waldemar Knittel, Germany). Sections were stained with hematoxylin-eosin (HE, Sigma), Massone Trichrome (DiaPath, Italy) and immunohistochemically using monoclonal mouse anti-human ready-to-use antibodies (Dako Omnis, Denmark) against Cytokeratin 7 (#clone OV-TL 12/30), Cytokeratin 20 (#clone KS 208), Ki-67 (#clone MIB-1), MUC5AC (#clone CLH2), mouse monoclonal anti-human TNF-alpha antibody (#clone DBM15.28, Diagnostic BioSystem, USA), and anti-CD45 (#clone EPR20033, Abcam). Before immunostaining, heat-induced antigen retrieval was performed with 20 min treatment in a PT Link (Dako Omnis) using EnVision Flex Target Retrieval Solution, High pH. After this, the slices were allowed to cool and sections were incubated for 30 min at room temperature with antibodies. For washing, EnVision Flex Wash Buffer was used, for visualization, an LSAB2 System-HRP has been applied using EnVision Flex DAB+ Substrate Chromogen System (Dako Omnis). Finally, slices were stained with Mayer hematoxylin. The samples were evaluated in Axiovert 200 light microscope (Zeiss).

Analysis of gene expression. Expression analysis of mRNA for human hTNFα gene and genes listed in Table 2 was determined by reverse transcriptase quantitative PCR (RT-qPCR). Total RNA was isolated from 5×10^5 cells or 5-50 mg of tissue using the innuPREP DNA/RNA Mini Kit (Analytik Jena GmbH, Germany), and RNA was depleted from genomic DNA by DNase treatment (DNase I, RNase-free; Thermo Fisher Scientific, USA). Next, 2 µg of total RNA was reverse transcribed using the SensiFAST cDNA Synthesis kit (Bioline, UK). Quantitative RT-qPCR was performed in triplicates using ampliTune® qPCR EvaGreen® Mix (Selecta Biotech SE, Slovakia), 250 nM concentration of primers, and 1 µl template cDNA per one reaction. The protocol for RT-qPCR was started with the activation step at 95 °C for 3 min, followed by 45 cycles of the denaturation step at 95 °C for 15 sec and annealing/polymerization at 60 °C for 15 sec with plate read steps at 75 °C. The PCR was performed in Bio-Rad 96FX cycler (Bio-Rad Laboratories, USA). The analysis was done using Bio-Rad CFX Manager software version 1.6 as normalized fold expression using the $2^{-\Delta\Delta Cq}$ method. The gene for hypoxanthine phosphoribosyl transferase 1

(HPRT1) was used as a reference. All oligonucleotides were synthesized by Metabion, Int. (Germany) or Sigma (USA).

Statistical analysis. The results are expressed as the mean±SEM or median+maximal value. Values were compared using a two-tailed Mann-Whitney U-test or a two-tailed Student's t-test in GraphPad Prism, version 6 (GraphPad Software Inc., USA). $P < 0.05$ was considered statistically significant.

Results

Tumor cells overexpressing TNFα differ in their ability to engraft in two different immunocompromised mouse strains. Engineered human tumor cell lines derived from colorectal carcinoma and melanoma, overexpressing the human TNFα gene (hTNFα), were used to induce subcutaneous tumors in two immunodeficient mouse strains. In athymic mice Balb/c-nu/nu (Figure 1A), the tumor cell engraftment of colorectal carcinoma cells HT-29hTNFα and melanoma cells A375hTNFα was completely disabled by TNFα overexpression (tumor take rate 0%). Mice were healthy, without any side effects caused by the transplantation of TNFα-overexpressing tumor cells for more than 90 days. Macroscopic examination revealed no pathological changes or metastatic foci in the internal organs (liver, spleen, lungs, colon). No enlargement of lymph nodes was detected. In one out of eight subcutaneous applications, small microvascularisation in the site of inoculation was observed.

In SCID/bg mice, tumor cells overexpressing hTNFα formed small flat ulcerous xenografts, prone to inflammation and later accompanied by necrosis and hemorrhage, confirmed by histologic examination (Figures 1C, 1D, 2C, 2D). Parental cells formed large xenografts, and mice were euthanized on day 29 (HT-29), 42 (HCT 116), and on day 26 (A375) for ethical reasons. On those days, hTNFα-engineered cells formed xenografts with significantly smaller volumes, reduced to 0-10% compared to xenografts derived from their respective parental cell lines (Figure 1B). After a prolonged time after inoculation, hTNFα xenografts started to grow, and the tumor take rate of transplanted HT-29hTNFα was 85.7% (12/14 xenografts), HCT 116hTNFα 50% (6/12 xenografts), and A375hTNFα cells 57% (8/14 xenografts) (Figure 1B). Parental tumor cells displayed tumor penetrance of 100% for HT-29 and A375 cells and 87.5% for HCT 116 cells. In 6 out of 7 mice injected with A375hTNFα cells, aggravation of health status was noticed, and cachexia developed in some animals. Severe life-threatening health complications developed in one mouse on day 29 (acute gastric dilation); the other two mice died on days 35 and 72. In one SCID/bg animal, impaired liver and enlarged spleen were identified as mouse spleen lymphoma after histologic and molecular examination. Xenografts of all parental cells formed large, rapidly growing tumors in

both immunodeficient mouse strains, and animals were euthanized on days 25-42, depending on the cell line.

Histopathological analysis revealed more differentiated tumor tissue in HT-29hTNFα xenografts. Hematoxylin-eosin staining of tumors derived from parental HT-29 cells revealed poorly differentiated adenocarcinoma with a partially glandular arrangement. The mitotic index varies, and there were many visible atypical mitoses. The stroma was formed by thin septa of collagen. In the center of the tumor, several necrotic areas merged and made up 40% of the entire area of the xenograft. The tumor infiltrated the surrounding tissue and skeletal muscle fibers. Xenografts derived from engineered HT-29hTNFα cells were well-differentiated adenocarcinomas, formed by variably large, branched glandular formations with several irregular, thicker projections into the lumen (Figure 2C). The tumor cell population consisted of eosinophilic cells with one darker, atypical or bizarre nucleus and bright cytoplasm. Along the periphery, several irregular glands infiltrated the surrounding sparse ligament. The mitotic index varies, and several atypical mitoses are visible. The stroma was formed by a thin ligament with mature collagen septa. Several necrotic foci with residual individual semi-vital tumor cells together made up 20% of the entire tumor area. In xenografts of engineered HT-29hTNFα cells, positivity for proliferation marker Ki-67 decreased to 20-50% in comparison to the control xenograft of parental HT-29 cells (70%). Large areas were negative for Ki-67, indicating a stromal fraction of the tissue of mouse origin. Positivity for CK7 localized in the periphery of the tumor decreased from 5% in parental xenografts to 0% in TNFα xenografts. A strong CK20 and MUC5A positivity of up to 100% was detected on the periphery of parental HT-29 xenografts, while in the center of the tumor, the positivity was 20-30%. In HT-29hTNFα tumors, the average positivity of CK20 and MUC5A markers was higher by 20% or more, as it is summarized in Table 3. We also observed a twofold increased percentage of collagen after Masson-trichrome staining (Figures 2C, 2D; Table 3).

Xenografts of parental cell line HCT 116 were of massive size and consisted of nodularly arranged poorly differentiated adenocarcinoma, partially with necrotic areas and pseudoglandular formations of variable size. Cells were closely arranged, with bright cytoplasm and darker heterochromatic nuclei and nucleoli. Xenografts of engineered cells HCT 116hTNFα were significantly smaller, poorly differentiated adenocarcinoma, infiltrating the skin. The TNFα tumors themselves underwent massive necrosis located centrally with a residual semi-vital population of tumor cells on the periphery (Figures 1D, 2D; Table 3), composed of pseudoglandular, pseudotubular formations and infiltrated the skin. Irregularly shaped cells had one darker, hyperchromic nucleus with several nucleoli and bright cytoplasm. Part of the tissue was formed by a tumor stroma, negative for Ki-67, CK7, and CK20. In the comparison of HCT 116 xenografts, in HCT 116hTNFα tumors the average

positivity for CK7 was markedly decreased. Positivity for CK20 disappeared in HCT 116hTNFα tumors. The HCT 116 cells are only weakly positive for CK20 and do not produce mucin MUC5A (Figure 2D; Table 3). In HCT 116hTNFα experimental tumors, a slight increase in collagen positivity was detected.

Immune cell infiltrates were stained using antibodies against pan-immune cell marker CD45. Xenografts of HT-29 and HT-29hTNFα cells showed 5% CD45 membrane-positive cells infiltrating from the tumor surface into the center of the tumor. In xenografts of engineered cells HCT 116hTNFα, significantly increased infiltration of CD45 membrane-positive cells was observed (up to 25% positivity) compared to 5% positivity in HCT 116 control xenografts. Immune cells were concentrated in the tumor surface capsule and then migrated from the surface into the center. In HCT 116hTNFα xenografts, strongly positive cells formed groups (Figures 2C, 2D; Table 3).

Expression analysis of HT-29 and HCT 116 derived xenografts overexpressing the TNFα gene revealed increased cytokeratin 20 and aldehyde dehydrogenase expression. Tumor tissues were analyzed for expression of human genes encoding TNFα, apoptosis-inducing ligand *TRAIL*, cytokeratins 20 and 7 (*CK20*, *CK7*), aldehyde dehydrogenases *ALDH1A1* (in HT-29 xenografts), *ALDH1A3* (in HCT 116 xenografts), and selected proteins of extracellular matrix: laminin gamma (*LAMC2*), collagen 1 (*COL1A1*) and collagen 4 (*COL4A4*). The results are summarized in Figures 2A and 2B.

The TNFα mRNA overexpression was confirmed in tumor tissues derived from engineered cells HT-29hTNFα and HCT 116hTNFα (Figures 1G, 1C; p=0.00953 for HCT 116), but the overexpression was also noticed in two of five xenografts of parental HT-29 cells (Ct 15.9 and 16.0 vs. 26-29 in other tissues). An increase of apoptosis-inducing ligand TRAIL mRNA expression was detected in HT-29hTNFα engineered cells *in vitro* [26], but it was not observed in their xenografts. Expression of CK20, a marker of colon epithelium differentiation, decreased in both TNFα-engineered xenografts (p=0.04670 for HT-29; p=0.04994 for HCT 116). The CK7 expression, a marker of poor differentiation and aggressiveness, was not changed significantly. Analysis of the expression of ECM proteins did not show changes in TNFα tumors, but significant overexpression of the aldehyde dehydrogenase gene was detected. The HT-29 cells are producers of the ALDH1A1 isoform, and the HCT 116 line are producers of the ALDH1A3 [27]. The RT-qPCR analysis showed increased mRNA expression of aldehyde dehydrogenase in TNFα engineered xenografts (p=0.05015 for *ALDH1A1* in HT-29 and p=0.01064 for *ALDH1A3* in HCT 116).

Discussion

Besides studying tumor and metastasis development, it is essential to explore ways how to modulate the TME into a tumor-suppressive state and stimulate the immune system to inhibit tumor growth. One potential tool remains the cytokine TNFα, which acts locally by modulating TME. TNFα stimulates key immune cells involved in antitumor surveillance, including CD8+ T cells, natural killer (NK) cells, dendritic cells (DC), and M1 macrophages. These cells are creating immunologically "hot" tumors, the tumor-suppressing inflammation, and TNFα is responsible for their recruitment, activation, and cytotoxicity [28].

In the presented study, colorectal cancer cells HT-29, and melanoma cells A375 engineered to overexpress TNFα completely lost their tumorigenicity in athymic nude mice. In SCID/bg mice, with no mature T and B cells and defective NK cells, identical cells HT-29, A375, and second CRC cell line HCT 116, formed rudimentary flat ulcerous xenografts with large necrotic lesions inside the tumor burden. Decreased tumorigenicity of engineered TNFα overexpressing cells and strong induction of necrosis are known and were described in several papers [10, 11, 13], and it was also reconfirmed by our study. The TNFα-overexpressing murine tumor cells administered systematically were homing to the primary tumor site, releasing low levels of TNFα, causing tumor apoptosis and tumor vasculature damage [9], serving as cell-directed mediators.

Some of the SCID mice bearing A375TNFα melanoma cells developed cachexia, similar to a previous study [29]. However, nude mice injected subcutaneously with A375TNFα cells remained healthy, showing no signs of cachexia. Mesenchymal stromal cells overexpressing TNFα, coinjected together with tumor cells, caused significantly reduced growth of melanoma xenografts, losing their tumor-supportive capacity [25]. Still, they could not suppress the development of experimental melanoma lung metastases [30]. TNFα-induced tumor necrosis is dependent on receptor TNFR1 signaling in the tumor vasculature [31]. Because human TNFα cannot bind to the mouse TNFR2 receptor [32], its activity is mediated exclusively via TNFR1 in *in vivo* experiments. The TNFR1 receptor, expressed ubiquitously across almost all cell types, contains a death domain capable of initiating apoptosis, necroptosis, or necrosis in mouse endothelial cells, mouse stromal fibroblasts, and human tumor cells within xenograft tissue. TNFα-mediated tumor suppression is also facilitated through STAT1-IFN-γ signaling within the nonmalignant components of the TME, mesenchymal stromal cells, and fibroblasts [33]. We also documented that in TNFα-overexpressing xenografts, $CD45^+$ leukocytes infiltrated from the periphery into the tumor center. The $CD45^+$ cells in immunodeficient SCID/Bg mice include myeloid cells – macrophages (TAMs), granulocytes (e.g., neutrophils), lymphoid- and myeloid-derived dendritic cells, and remaining NK cells, which are defective but still exist in SCID/Bg mice. All these cells are also able to produce TNFα. The highest proportion of $CD45^+$ cells are TAMs, which are also highly responsive to TNFα. In

immunohistochemical evaluations of CRC in patients, 40-44% of TAMs are M2-like, while a subset of M1-like TAMs is less prevalent in tumors compared to adjacent normal mucosa [34], so we can conclude that most of the observed $CD45^+$ cells in xenografts are TAMs. Our study, employing two different immunodeficient mouse strains, suggests that the antitumor activity of TNFα in creating a tumor-resistant microenvironment probably depends on the presence of NK cells and B cells, which are still present in nude mice. There is relatively little information about the antitumor antibody response mediated by B cells, but TNFα can help to transmit a signal from tumor-binding IgG for presenting the tumor antigen by dendritic cells (DC), and start IgG-activation of T cells [35]. Potential antitumor effect via membrane TNFα can mediate activation of cytotoxic action of B cells [36]. The apoptotic killing and fragmentation of cancer cells may be then an initial step in tumor antigen uptake and presentation by DCs and other cells of antitumor immune response.

We also consider the differences in the organization of human and mouse immune systems, and that the mouse models of cancer are artificial. Mice are more tolerant to lipopolysaccharides, systemic inflammation, and the effects of cytokines in comparison to humans. Doses of lipopolysaccharides inducing severe disease with shock are several orders of magnitude higher in mice than in humans. The mouse can also reduce the TNFα production very efficiently. However, the human and murine NK cells show similarities in cytokine responsiveness. The signaling pathways are largely conserved in NK cells [37, 38], and the TNFR1-TNFα signaling significantly activates the antitumor activity of NK cells. In the mouse, selective NK cell deficiency has been associated with a failure to reject tumor cells [39].

It is known, that xenografts of the HT-29 adenocarcinoma cell line exhibit the ability to differentiate and form enterocyte-like and mucin-producing structures under specific conditions. Histological analysis of xenografts of engineered HT-29TNFα cells revealed a more differentiated phenotype with clearly visible pseudoglandular structures compared to apparently poorly differentiated control xenografts. Tumors with more differentiated phenotypes have a better prognosis, while immature, undifferentiated tumors are more aggressive. We also detected a more abundant mouse stromal fraction within the tumor burden and decreased positivity for the Ki67 proliferation marker in engineered xenografts.

The standard phenotype of colorectal carcinomas is CK20 positivity and CK7 negativity, but there have been described several IHC pattern which are significant in tumor differentiation, including metastatic potential [40]. Nevertheless, CK7 is an important marker of metastatic CRC. CRC patients with CK7-positive tumors had shorter 5-year survival, indicating a negative prognostic role of CK7 [41]. Xenografts in our study were also positive for CK7, and the TNFα overexpression was linked with a decreased CK7 positivity. The loss of CK20 expression is associated with a

significantly favorable prognosis of CRC in another survival study [42], and our experimental tumors overexpressing TNFα displayed decreased expression of CK20, too.

Aldehyde dehydrogenase (ALDH) is overexpressed in various tumors and considered an important CSC marker, linked with multidrug resistance, poor prognosis, and is a possible marker of cancer stem cells [43]. Our results show increased *ALDH1* expression in the xenograft tissue of both CRC lines producing TNFα (isoform *ALDH1A1* for HT-29 and *ALDH1A3* for HCT 116). However, various effects of TNFα on the ALDH enzyme have been published: a decrease of ALDH enzyme activity in identical TNFα-engineered tumor cells *in vitro* [26], no effect in malignant cells, and also an increased ALDH activity in human bone marrow cells [44], and in breast carcinoma cell line [45] treated with TNFα. We did not observe increased expression of human *TRAIL in vivo*, which was induced in identical cells *in vitro* [26]. Overexpression of *TNFα* was also noticed in parental HT-29 xenografts, probably driven from the innate gene. Grimm et al. [46] also reported high TNFα protein expression in the HT-29 cell line itself and demonstrated that elevated TNFα levels were associated with worse prognosis and the presence of lymph node metastases in CRC patients. So, besides immune cells, even malignant cells themselves can be producers of TNFα. Extracellular matrix, important in TME and in the prognosis of disease, was not changed significantly under TNFα secretion in CRC line xenografts, as shown by Masson's Trichrome staining and PCR analysis. We noticed only a slight increase in collagen positivity.

In the present study, we document several changes initiated by the TNFα production *in situ* in experimental model: disabled cancer cell engraftment and zero tumorigenicity in the presence of NK and B cells (nude mice), significantly reduced growth of tumors in the absence of T, B, and NK cells (SCID/bg mice), increased immune cells infiltration, better-differentiated tumor tissue organization, decreased positivity for cytokeratins 7 and 20 and increase of expression of ALDH1 isoforms. Our study suggests that the tumor microenvironment can be effectively modulated by TNFα, and controlled stimulation of antitumor immunity may be beneficial for future cancer treatment strategies.

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Figure Legends

Figure 1. Overexpression of TNFα affects tumorigenicity and differentiation in colorectal cancer xenografts. A) Plots of individual tumor growth in Balb/c-nu/nu mice injected *s.c.* with 5×10^5 A375 (n=8), 2.5×10^5 HT-29 cells (n=8), and comparison of xenograft size: A375 on day 23 and HT-29 on day 24. B) Individual tumor growth in SCID/bg mice injected *s.c.* with 5×10^5 A375 cells (n=10 and 20), 2.5×10^5 HT-29 cells (n=8), 2.5×10^5 HCT 116 cells (n=8 and 12) and comparison of xenograft size: A375 on day 25-26 (n=10 and 12), HT-29 on day 29 (n=8), HCT 116 on day 42 (n=8 and 12). C) Macroscopic view of *s.c.* of A375 overexpressing TNFα on day 61 (upper images) and HT-29 overexpressing TNFα on day 55 (lower images), approx. size of 2-4 mm in diameter, SCID/bg mice. D) Massive necrosis inside the HCT 116hTNFα xenograft and semivital tumor cells localized peripherally; HE staining, magnification 5×.

Figure 2. Histopathological changes in xenografts of TNFα overexpressing colorectal cancer cells in SCID/bg mice: better tissue differentiation and CD45 positive immune cell infiltration. Expression analysis of tissues derived from HT-29 (A) and HCT 116 cells (B): mRNA expression of *TNFα*, apoptosis-inducing ligand *TRAIL*, collagen 1 (*COL1A1*), collagen 4 (*COL4A4*), laminin γ (*LAMC2*) and aldehyde dehydrogenase isoforms 1A1 and 1A3 (*ALDH1A1*, *ALDH1A3*) and cytokeratins CK7 and CK20; RT-qPCR, 2 x 2 factorial model on ΔCt values analyzed per gene, P values from two-sided t-test, data are shown as individual data points for individual xenografts and mean ± SD. C) Histological and IHC analysis of xenografts derived from HT-29 cells; HE: HT-29 – poorly differentiated adenocarcinoma, HT-29hTNFα – moderately differentiated carcinoma with pseudoglandular structures, Masson's Trichrome staining for connective tissue (blue) and epithelial tissue (red), IHC for TNFα, CD45, Ki67, CK7, CK20, Mucin glycoprotein MUC5A; xenografts were induced by 2.5×10^5 cells in SCID/bg mice. Magnification 20×. D) Histological and IHC analysis of xenografts of HCT 116 cells; HE of poorly differentiated adenocarcinomas, Masson's Trichrome staining for connective tissue, IHC for TNFα, CD45, Ki67, CK7, CK20; xenografts were induced by 2.5×10^5 cells in SCID/bg mice; red arrow – necrosis, blue arrow – stromal cells, black arrows – pseudoglandular structures; magnification 20×.

535 **Table 1.** The difference between immunodeficient mice strains SCID/bg and Balb/c-nu/nu and their immune system [47, 48].

	Inbred Nude – Balb/c-nu/nu	**Fox Chase SCID Beige – SCID/bg**
Genetic mutations	disruption of FOXN1 gene encoding DNA-binding transcription factor on chromosome 11, regulating cytokeratins involved in the development of thymic epithelium	autosomal recessive mutations SCID (Prkdcscid) and Beige (Lystbg) deficient activity of an enzyme involved in DNA repair and antigen-receptor gene assembly
Result of mutations	dysgenesis of thymic epithelium and defect in helper T-cell activity	inability of T and B cells to mature and in complete loss of adaptive immunity
Mature T cells	absent	absent
Mature B cells	present	absent
Dendritic cells	present	present
Macrophages	present	present
Natural Killer cells	present	defective
Leakiness	N/A	low
Consideration	intact innate immunity little engraftment of hematopoietic cancer cells not suitable for primary cells	defective NK cells due to beige mutation
Engraftment of TNFα-producing tumor cells	failed	successful with delayed growth

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537 **Table 2.** List of primers used for RT-qPCR or qPCR.

Gene	Forward primer sequence 5'→3´	Reverse primer sequence 5'→3´	Amplicon length (bp)
TNFα	CCTCAGCCTCTTCTCCTTCC	AGATGATCTGACTGCCTGGG	145
TRAIL	ACCAACGAGCTGAAGCAGAT	ACGGAGTTGCCACTTGACTT	141
CK20	AGACACACGGTGAACTATGGG	CTCCAGGGTCCGCACCTTTT	128
CK7	AGTGTCCCCGAGGTCAGCGA	AGAGGCTGCTGCTGCCAAGG	187
ALDH1A1	CAACAGAGGTTGGCAAGTTGATC	CATGGTGTGCAAATTCAACAGC	141
ALDH1A3	TCTGGAACGGTCTGGATCAACTGC	CCTTTCCTTCAGGGGTTCTTGTCG	171
LAMC2	GGATTCAGTGTCTCGGCTTC	TGCTGTGCTTCTTCTTTCCA	168
HPRT1	GACCAGTCAACAGGGGACAT	CCTGACCAAGGAAAGCAAAG	132

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539 **Table 3.** Histopathological characteristics of xenografts of parental colorectal cancer cells and xenografts of cells engineered to overexpress TNFα.

xenograft	**HT-29**	**HT-29hTNFα**	**HCT 116**	**HCT 116hTNFα**
tumor cell arrangement	low-differentiated infiltrative adenocarcinoma	well-differentiated infiltrative adenocarcinoma	low-differentiated adenocarcinoma	low-differentiated adenocarcinoma
necrosis	0-20%	40%	55%	60%
necrosis in center	10%	90%	80%	95%
necrosis in periphery	10%	10%	20%	5-10%
TNFα positivity	80-90%	80-90-100%	5-10%	5%
Ki-67 positivity	70%	20-50%	70-80%	70-80%
CD45 positivity in center	< 5%	< 5%	< 5%	5-25%
CD45 positivity in periphery	< 5%	5-25%	5-25%	5-25%
CK7 positivity	5%	0%	50-70%	5-20%
CK20 positivity	60%	70-80%	0-5%	0%
MUC5A positivity	50%	60-80%	0%	0%
Masson Trichrome positivity (collagen positivity)	10%	20-25%	5%	5-10%

540

Fig. 1 [Download full resolution image]

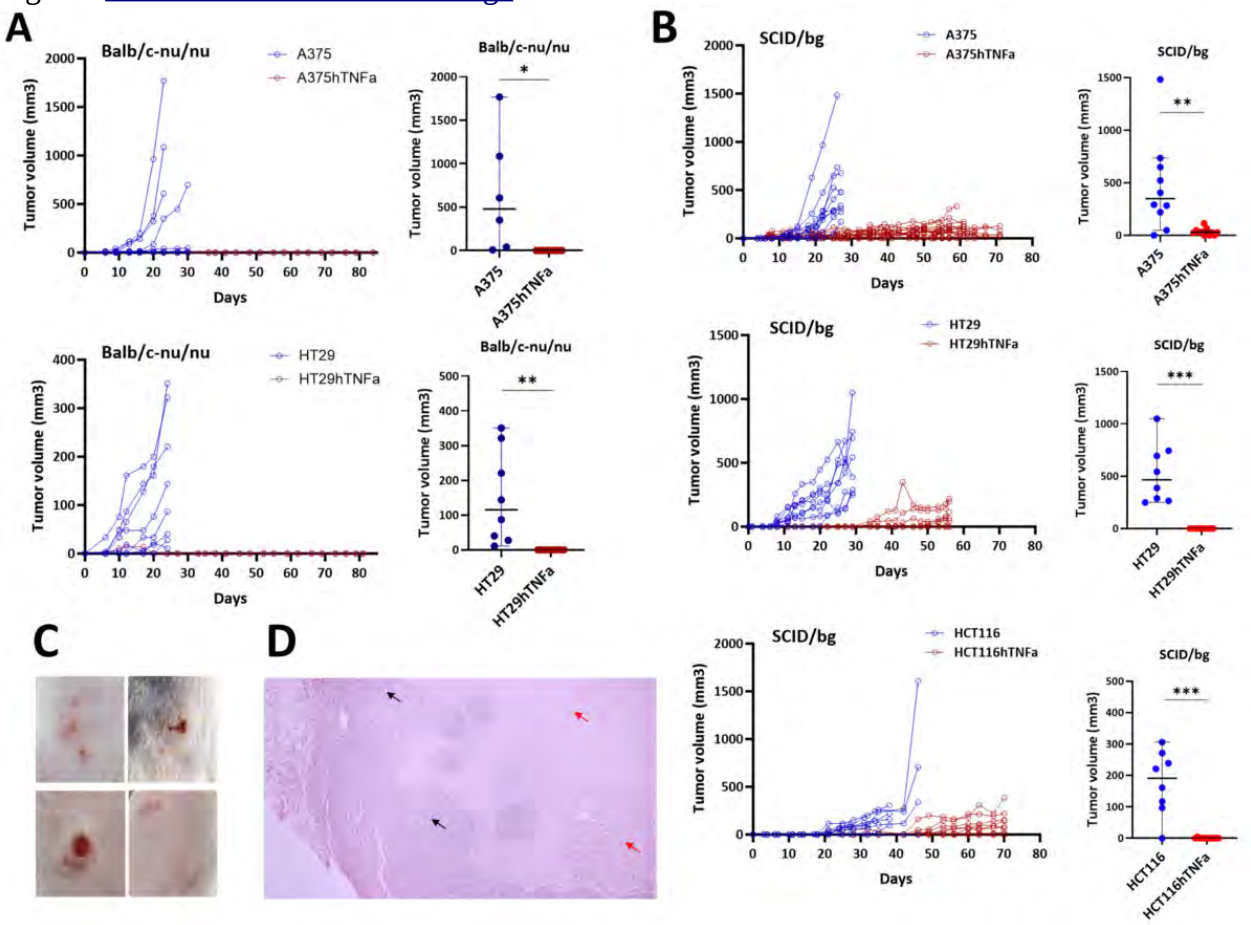

Fig. 2 Download full resolution image