

Colon Cancer Stem Cells Dictate Tumor Growth and Resist Cell Death by Production of Interleukin-4

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SUMMARY

A novel paradigm in tumor biology suggests that cancer growth is driven by stem-like cells within a tumor. Here, we describe the identification and characterization of such cells from colon carcinomas using the stem cell marker CD133 that accounts around 2% of the cells in human colon cancer. The CD133⁺ cells grow *in vitro* as undifferentiated tumor spheroids, and they are both necessary and sufficient to initiate tumor growth in immunodeficient mice. Xenografts resemble the original human tumor maintaining the rare subpopulation of tumorigenic CD133⁺ cells. Further analysis revealed that the CD133⁺ cells produce and utilize IL-4 to protect themselves from apoptosis. Consistently, treatment with IL-4R α antagonist or anti-IL-4 neutralizing antibody strongly enhances the antitumor efficacy of standard chemotherapeutic drugs through selective sensitization of CD133⁺ cells. Our data suggest that colon tumor growth is dictated by stem-like cells that are treatment resistant due to the autocrine production of IL-4.

INTRODUCTION

Colorectal cancer is the third most common visceral malignancy and remains one of the leading causes of cancer-related deaths due to therapy resistance (Greenlee *et al.*, 2001). Normal homeostasis in the intestinal tract is regulated by a careful balance between cell proliferation, differentiation, and apoptosis. Although this balance is clearly disturbed in colorectal cancer, closer examination of tumors from the colon reveals that there is heterogeneity within such tumors that may in part be due to (aberrant)

differentiation of cancer cells. Until a few years ago, all neoplastic cells within a tumor were suggested to contain tumorigenic growth capacity, but recent evidence hints to the possibility that this is confined to a small subset of cancer-initiating cells, so-called cancer stem cells (CSC) (Pre-snell *et al.*, 2002; Reya *et al.*, 2001). The first evidence for the existence of CSC was obtained in the context of acute myeloid leukemia, where only a small portion of harvested leukemic cells from patients could recapitulate the same cancer in immune-deficient mice (Bonnet and Dick, 1997). Later on, CSC were also demonstrated to exist in breast (Dontu *et al.*, 2004) and brain tumors (Singh *et al.*, 2004). These cells express markers of stemness and are also capable to reproduce the human cancer in mouse models. Other reports extend this principle to prostate (Collins *et al.*, 2005), ovarian carcinoma (Bapat *et al.*, 2005), and melanoma (Fang *et al.*, 2005). More recently, two reports showed the existence of CSC in colon carcinoma using CD133 as a marker (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007). CD133 is infrequently expressed in normal colon tissues as compared with the tumor tissues (O'Brien *et al.*, 2007). These reports suggest that colon cancer is organized in a hierarchical fashion in which the CD133⁺ tumor cells act as stem-like cells and thereby give rise to more differentiated tumor cells. However, the nature of these undifferentiated colon cancer cells and the sensitivity of these cells for treatment requires further investigation.

CSC are hypothesized to be inert to toxic environmental agents due to high expression levels of ABC transporters, active DNA-repair capacity, and resistance to apoptosis (Dean *et al.*, 2005). Alterations in the apoptotic machinery have been related to chemoresistance in several tumor types (Johnstone *et al.*, 2002). We recently described the resistance of cancer cells to apoptosis due to elevated expression of antiapoptotic proteins, associated to the autocrine production of IL-4 (Stassi *et al.*, 2003; Todaro *et al.*, 2006). IL-4 has previously been reported to induce apoptosis resistance in chronic lymphocytic leukemia B cell and to enhance antiapoptotic protein expression in

normal and transformed lymphocytes (Dancescu et al., 1992), as well as in prostate, breast, and bladder tumor cell lines (Conticello et al., 2004). Additionally, it has been demonstrated that IL-4 enhances proliferation of human pancreatic cancer cells, whereas blockage of this cytokine on these tumor cells has a significant growth-inhibitory effect (Prokopchuk et al., 2005). Thus, it is likely that IL-4 may promote cancer cell survival through upregulation of antiapoptotic genes. Given this evidence, we defined the tumorigenic stem-like cell population present in colorectal carcinoma and analyzed whether this population is more resistant to current therapies. Moreover, we analyzed whether CSC can be sensitized to undergo growth inhibition and cell death by neutralizing the IL-4 pathway.

RESULTS

Colon Cancer Contains Stem-like Cells

Several colon cancer specimens were characterized by alcian blue and hematoxylin and eosin stainings; by immunohistochemistry for CK20, CK7, the Ki67 antigen and the homeodomain transcription factor CDX2 (Figure 1A); and were typed for microsatellite instability (MSI) (Table 1). Moreover, these tumors were analyzed for the expression of CD133, a putative stem cell marker. Five (24%) tumors from our cohort were MSI-high (MSI-H), six (28%) tumors were MSI-low (MSI-L), and nine (48%) lacked MSI (MSS) (Table 1). The CD133 cell antigen, which is expressed on hematopoietic stem cells (Yin et al., 1997) and recently used to identify brain (Singh et al., 2004), kidney (Bussolati et al., 2005), colon (O'Brien et al., 2007; Ricci-Vitiani et al., 2007), and prostate cancer stem-like cells (Collins et al., 2005), was detected within the tumor specimens as well (Figure 1A,B). A further analysis achieved on freshly purified cells from these tumors revealed that the percentage of CD133⁺ cells in all patients ranges between 0.3%–3% (Table 1 and Figure 1C left panel).

CD133⁺ Cells Mediate Tumor Growth in Xenotransplants

To determine whether such CD133⁺ cells have tumorigenic capacity and thus, in analogy with previous findings, are required for successful xenotransplantation, we digested primary tumor samples and analyzed their ability to grow subcutaneously in immunodeficient mice. Freshly dissociated cells from human cancer specimens (bulk) injected at 0.5 or 2 × 10⁶ cells per mouse were capable of establishing subcutaneous tumors (Figure 1D). However, depletion of the CD133⁺ cells (≤0.08%; Figure 1C) from the same tumor samples using magnetic bead cell sorting drastically reduced tumor formation (Figure 1D). The failure of the CD133⁻ population to form tumors after xenotransplantation was not due to a complete depletion of cancer cells during the cell sorting procedure, as more than 60% of the remaining CD133⁻ cells were mutant for P53 in four screened tumors (see Figure S1A in the Supplemental Data available with this article online) and both p53 and *k-ras* mutations were detected in the

CD133⁻ cells (Table S1). These observations confirm that CD133⁻ tumor cells do not induce tumor formation, while as little as 2.5 × 10³ or 5 × 10³ of the purified CD133⁺ cells, either derived from MSI or MSS tumors, readily form tumors (Figure 1D).

The xenografts tumors that resulted from injection of CD133⁺ cells presented the same histopathology features as their respective human tumor and were composed of CD133⁺/CD133⁻ ratios similar to that observed in the original tumors.

Taken together, our data confirm recent observations indicating the potential of CD133⁺ cells to drive tumor growth (O'Brien et al., 2007; Ricci-Vitiani et al., 2007).

Spheroid Cultures of Colon Cancer Stem-like Cells

In an attempt to keep the colon cancer cells in culture for additional characterization, freshly purified cells were seeded under standard adherent conditions. These cultures were all characterized by clusters of polygonal cells (Figure 2A, left panels) that grew up to about 2 weeks. The adherent cells were positive for CDX2, cytokeratin-AE (CKAE), CK20, and β-catenin, whereas CK7 was undetectable, which is a typical pattern for colorectal cells (Figure 2A, left panels). Intriguingly, none of the cells in such culture conditions was positive for CD133 (Figure 2D and data not shown). In contrast, when colon carcinoma samples from the same patients were dissociated into single cells and cultured in a serum-free medium containing EGF and FGF-2, a sphere-like culture was obtained with a frequency of 10 out of 21 tumors (45%) (Table 1; Figure 2B, left panel). The sphere-like aggregates could be expanded for several months in this medium in line with previous reports on neural and mammary stem cells (Dontu et al., 2003; Singh et al., 2004). Spheroid growth appeared to be more effective when using MSI tumors, but is not exclusive for such tumors (Table 1).

These spheroid cultures were all characterized by cells with round shape that were negative for CDX2, CK20, and CK7, but positive for CD133 (Figures 2A and 2B). As assessed by FACS analysis, about 90% of the spheroid cells were positive for CD133 protein (Figure 2C). Further immunoblot analyses assessed on control, cancer adherent cells and cancer spheres confirmed the expression of CD133 on cancer spheroid cells (Figure 2D), indicating that spheroid cultures contain cells that have the same cell surface marker as the CSC observed within the original tumor. In agreement, these spheroid cultures can be directly derived from the CD133⁺ colon cancer cells as purified CD133⁺ cells from 5 out of 21 different tumor specimens formed spheroids and grew exponentially as undifferentiated cells. This latter capacity seems to correlate with MSI-high tumors, although it is also observed with one MSS colon carcinoma (Table 1).

Spheroid cultures that were supplemented with fetal bovine serum (FBS) for up to 21 days initially displayed adherence to the culture dishes and subsequently differentiated into large, polygonal colon cells starting from week 1, a phenotype similar to that of the adherent primary cultures (Figure S2A).

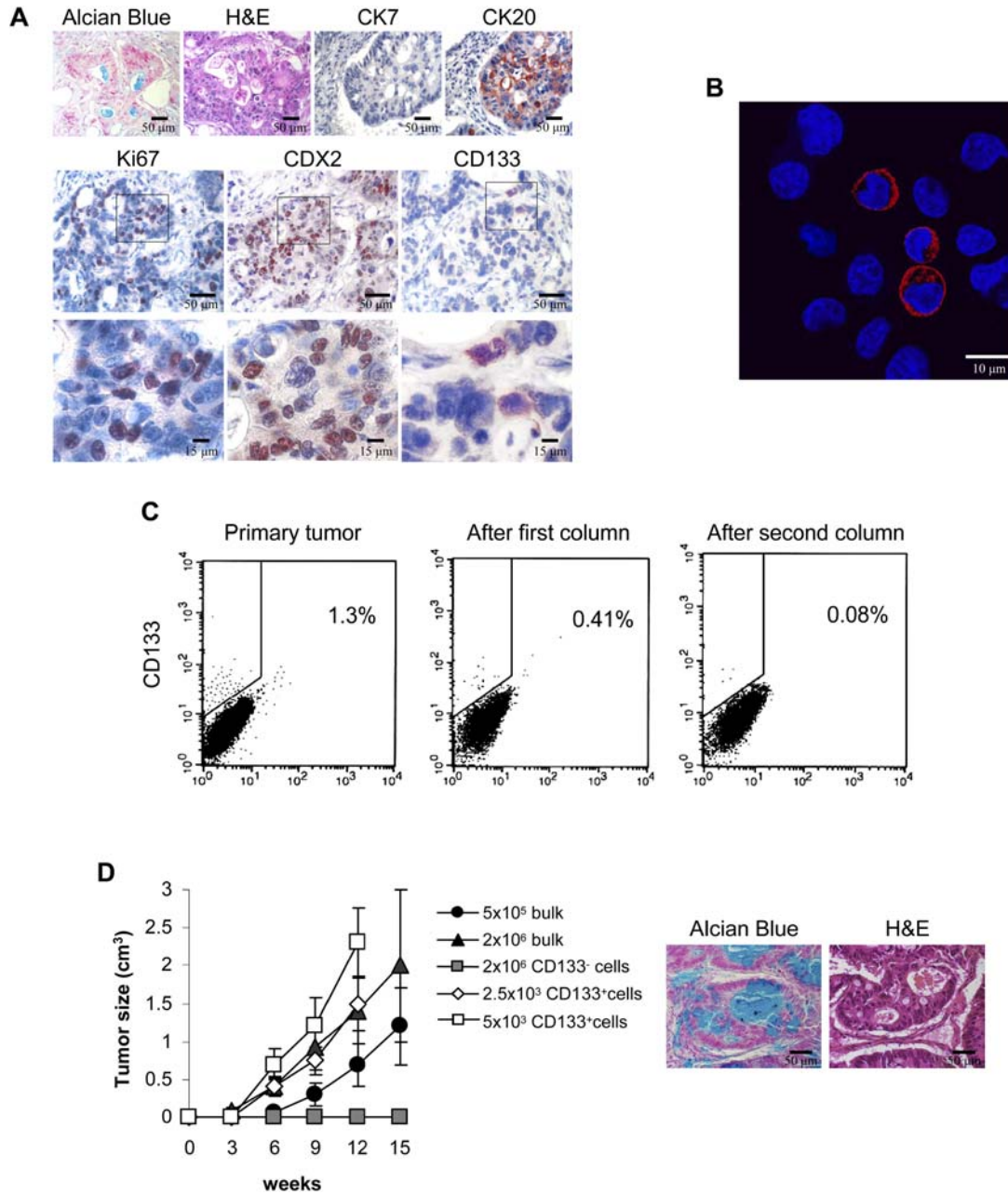


Figure 1. Colorectal Cancer CD133⁺ CSC

(A) Alcian blue, H&E stainings, and immunohistochemical analysis of CK7, CK20, Ki67, CDX2, or CD133, revealed by AEC (red staining), on paraffin-embedded sections of colorectal cancer. Nuclei are revealed by hematoxylin staining (blue). One representative of nine different tumors is shown. (B) Confocal microscopy analysis of CD133 on freshly purified bulk cells from colon cancer specimen. Nuclei were counterstained by Hoechst (blue color). (C) Flow cytometry analysis for CD133 performed on CD133-depleted colon cancer cells using magnetic beads. Data are representative of 21 tumors analyzed.

(D) In vivo growth of colon carcinoma tumor cells. Colon carcinoma specimens were digested and colon cancer cells were either injected directly into the right flank of mice (closed triangles and circles) or first magnetically separated for CD133 positive (open squares and diamonds) and depleted of CD133 cells (gray square) and then injected. Data are mean tumor size ± SD of 15 tumors per group derived from three independent experiments from different patients. Histology panels represent Alcian blue and H&E staining of xenografted tumors and confirm a colorectal cancer phenotype.

More importantly, when colon cancer spheroids were cultured in the presence of FBS in matrigel, which represents a reconstituted 3D culture system, colonies that were generated were organized in a complex structure

reminiscent of a colonic crypt (Figure 2E; Figure S2B, upper panels), whereas primary cancer cells from the adherent cultures failed to form these structures (Figure S2B, lower panels). These crypt-like structures were positive

Table 1. Case Description and Tumor Features

Patient	Age/Sex	Site	Dukes	Spheres Formation	CD133 (%)	MSI	CD133-Derived Spheres	Anti-IL-4 Sensitization
P1	65/M	recto-sigmoid	B	yes	1.8	MSI-L	no	yes
P2	70/M	sigma	C	no	0.3	MSS	no	nt
P3	55/F	left	A	no	1.2	MSS	no	yes
P4	68/F	sigma	B	no	1	MSS	no	nt
P5	66/F	recto-sigmoid	C	yes	1.4	MSI-L	no	yes
P6	58/M	sigma	A	no	0.8	MSS	no	nt
P7	65/F	sigma	D	yes	1.3	MSI-H	yes	yes
P8	57/F	left	D	yes	1.5	MSI-H	yes	yes
P9	73/F	right	B	no	0.3	MSS	no	yes
P10	63/M	recto-sigmoid	B	yes	1.9	MSI-H	no	yes
P11	76/M	right	B	no	3	MSI-L	no	yes
P12	70/F	sigma	A	no	1	MSS	no	yes
P13	68/M	recto-sigmoid	D	yes	0.9	MSI-L	no	no
P14	57/M	recto-sigmoid	C	yes	1.3	MSI-H	yes	yes
P15	76/F	sigma	C	no	1	MSS	no	yes
P16	56/F	left	B	no	2.1	MSI-L	no	yes
P17	75/F	left	C	no	1.8	MSS	no	no
P18	74/F	sigma	D	no	1.2	MSS	no	yes
P19	56/F	sigma	D	yes	1.5	MSI-L	no	yes
P20	68/M	recto-sigmoid	D	yes	1.7	MSI-H	yes	yes
P21	56/F	left	D	yes	2.4	MSS	yes	yes

Flow cytometry analysis for CD133 was performed on freshly purified cells from human colon tumors.

for alcian blue, CK20, and β -catenin, while CD133 expression was gradually lost (Figure 2E). We therefore conclude that colon cancer spheroids represent a subpopulation of cells that contain stem-like features and have the capacity to differentiate in vitro into luminal structures.

Colon Spheres Can Generate Tumors upon Xenotransplantation

Subcutaneous injection of low numbers of spheres confirmed that the spheroid cells retain the capacity to initiate tumor growth in nude mice (Figure 2F; Figure S2C; Table 2). As little as 500 spheroid cells were sufficient to obtain tumor growth (Figure S2C), while 2×10^6 adherent cells were ineffective (Figure 2F). Histological examination of xenografts derived from spheroid cultures showed that these tumors closely resemble the original human tumor mainly containing differentiated cells and a small fraction of stem-like cells expressing CD133 marker (Figure S2D). Importantly, spheroid cultures can be rederived from the xenotransplanted tumors (Figure S2E), and these retain the stem-like features as they will form tumors that have the original morphology upon reinjection (Figure S2F). These data therefore indicate that the CD133⁺ cells within colon carcinoma can be propagated

in spheroid cultures, represent cancer stem-like cells that contain tumorigenic capacity, can be reisolated and transplanted from xenografts, and are able to differentiate in vitro and in vivo.

CD133⁺ Cells Are Resistant to Cell Death Due to IL-4 Production

Since cancer stem cells are thought to be particularly resistant to drug treatments, we analyzed the cell viability of primary colon cancer cells following exposure to oxaliplatin and/or 5-fluorouracil (5-FU) at clinically relevant doses or after exposure to the death ligand TRAIL.

Cancer cells or purified CD133⁻ cells from the same human colon cancer specimens showed a high sensitivity in vitro to both drugs tested in a dose-dependent fashion (Figure 3A). The observed sensitivity of the CD133⁻ cells was not due to death of nontumor cells within this subpopulation as a clear reduction in the number of P53 mutant cancer cells was detected (Figure S1B). In contrast, CD133⁺ cells were largely inert to chemotherapeutic drugs-induced apoptosis even at the highest dose concentrations used (Watanabe et al., 2006). The ED₈₀ values indicate an approximate 60- and 15-fold difference for 5-FU and oxaliplatin, respectively (Figure 3A).

Previously, we described that autocrine production of IL-4 strongly modulates death receptor- and chemotherapy-induced apoptosis (Stassi et al., 2003; Todaro et al., 2006). Receptors for IL-4 are expressed on hematopoietic cells and a range of nonhematopoietic cells including epithelial, endothelial, muscle, fibroblast, and liver cells. On nonhematopoietic cells, the receptor complex for IL-4 (type II) is predominantly composed of the IL-4R α and IL-13R α 1 chains which heterodimerize upon either IL-4 or low-affinity IL-13 binding (Mentink-Kane et al., 2004). Another glycoprotein chain implicated in the IL-4R system, but with higher affinity for IL-13, is the IL-13R α 2 chain described to act as strong inhibitor of the IL-13 signaling by functioning as a decoy receptor (Rahaman et al., 2002). Intriguingly, colon cancer samples clearly displayed high levels of IL-4 expression (Figures 3C–3F; Table S2), which was absent in normal colon (Figure 3C). In addition, IL-4-receptor (IL-4R α) expression was also detected in colon cancer specimens (Figure 3C). IL-4 and IL-4R α were also expressed on cells of spheroid cultures and on freshly purified CD133⁺ cells (Figures 3C–3F). In contrast, the IL-4-related cytokine, IL-13, was undetectable on normal and colon cancer epithelial cells, neither on CD133⁺ nor in cancer spheroid cells (Figures 3G and 3H; Figure S1C; Table S2), while it was expressed on smooth muscle and infiltrating gut lamina propria cells of the normal mucosa (data not shown) (Morimoto et al., 2006). IL-13R α 2, the decoy for IL-13, was clearly present in colon epithelial cells, but higher in normal than in colon cancer cells (Figures 3G and 3H). Combined, these data suggest that colon cancer cells may display autocrine IL-4 signaling.

To investigate whether IL-4 constitutes a survival factor for CD133⁺ cells, we pretreated freshly isolated cells with IL-4-neutralizing antibody, and then drug resistance of CD133⁺ cells was evaluated. When anti-IL-4 antibody was followed by oxaliplatin, 5-FU or TRAIL treatment resulted in a significantly enhanced overall cell death rate (Figure 3B). Best results were obtained when anti-IL-4 was combined with either oxaliplatin plus 5-FU ($p < 0.001$) or oxaliplatin alone ($p < 0.001$) with no significant difference between both treatments (Figure 3B). Similarly, anti-IL-4-induced sensitization to drugs was also observed in the long-term spheroid cultures (Figures 4A and 4B), and even anti-IL-4 antibody alone showed a small but consistent decrease in the viable cell number (Figure 4A), suggesting that IL-4 is required for the survival of CD133⁺ cells. Mechanistically, we found that anti-IL-4 treatment resulted in a decreased expression of the antiapoptotic proteins cFLIP, Bcl-x_L, and PED by about 2-fold (Figure 4C), which may point to an IL-4-induced antiapoptotic program. These findings indicate that IL-4 protects CD133⁺ cells and could thus dictate therapy refractoriness, possibly through the upregulation of antiapoptotic proteins.

IL-4 Inhibition Enhances Tumor Response to Oxaliplatin and 5-FU

To test this hypothesis in vivo, we analyzed whether IL-4 blockade enhances chemotherapy efficacy. Since IL-4

and IL-13 share a common receptor subunit (IL-4R α), an efficient way to block cytokine-induced proliferation in xenotransplants would be to disrupt ligands/receptor interaction. We used a high affinity antagonist of IL-4R α characterized by amino acid replacements in two different positions of the IL-4 molecule (IL-4DM) as strategy to inhibit both IL-4 and IL-13 responses (Grunewald et al., 1998).

Colon cancer spheroids were allowed to grow into the flank of nude mice until they reached a size of ~ 0.2 cm³. Tumors formed had the morphological characteristics of adenocarcinomas and mainly contained CD133⁺ cells, whereas only a minority of CD133⁺ was present. Mice were then treated intraperitoneally (i.p.) twice a week for 3 weeks with IL-4DM or with PBS starting 24 hr before drug delivery (Figure 5A). In all treated mice, the tumor response to chemotherapeutic drugs was enhanced by IL-4DM. Specifically, IL-4DM cotreatment with oxaliplatin or oxaliplatin plus 5-FU resulted in a marked synergistic effect on tumor control, whereas a less pronounced effect was observed with 5-FU and IL-4DM alone (Figure 5A; Figure S1D).

As the colon cancers examined were not producing IL-13, we set out to confirm the role of IL-4 directly with the use of intratumoral injection of neutralizing antibody against IL-4 (twice a week for 3 weeks). PBS and control antibody treatment alone did not prevent tumor outgrowth, and anti-IL-4 only had a marginal effect (Figure 5B). As observed before, intraperitoneal treatment with oxaliplatin alone delayed tumor growth by about 4 weeks, but when treatment was interrupted, tumor growth was as rapid as in the sham-treated mice, as shown by the steepness of the growth curves (Figures 5A and 5B). By contrast, cotreatment with anti-IL-4 strongly enhanced tumor response to chemotherapeutics, similar to the IL-4DM effect (Figure 5B).

This data suggests that although standard chemotherapeutic drugs delay tumor outgrowth during the treatment, when they are combined with IL-4DM or with IL-4 neutralizing antibody, the efficacy of chemotherapy is significantly enhanced and also more sustained after treatment interruption (Figures 5A and 5B).

Anti-IL-4 Sensitizes Cancer Stem-like Cells

The mechanism of in vivo cell death sensitization of cancer cells to drugs-induced apoptosis was evaluated using immunohistochemical analysis of anti-IL-4 treated tumors. TUNEL staining, which reveals apoptotic cells, several days after the last oxaliplatin treatment, confirmed that anti-IL-4 plus oxaliplatin resulted in a significant higher number of apoptotic events compared to oxaliplatin treatment alone (Figure 5C). TUNEL costaining with CD133 to identify apoptosis in the stem cell compartment is feasible and showed TUNEL positivity in CD133⁺ cells in vivo only when tumors were cotreated with oxaliplatin and anti-IL-4 and not when they were treated with oxaliplatin and control antibody (Figure 5D). This suggests that CD133⁺ cells are also in vivo more resistant than other cancer cells to drug treatment. In accordance, treatment of xenografts

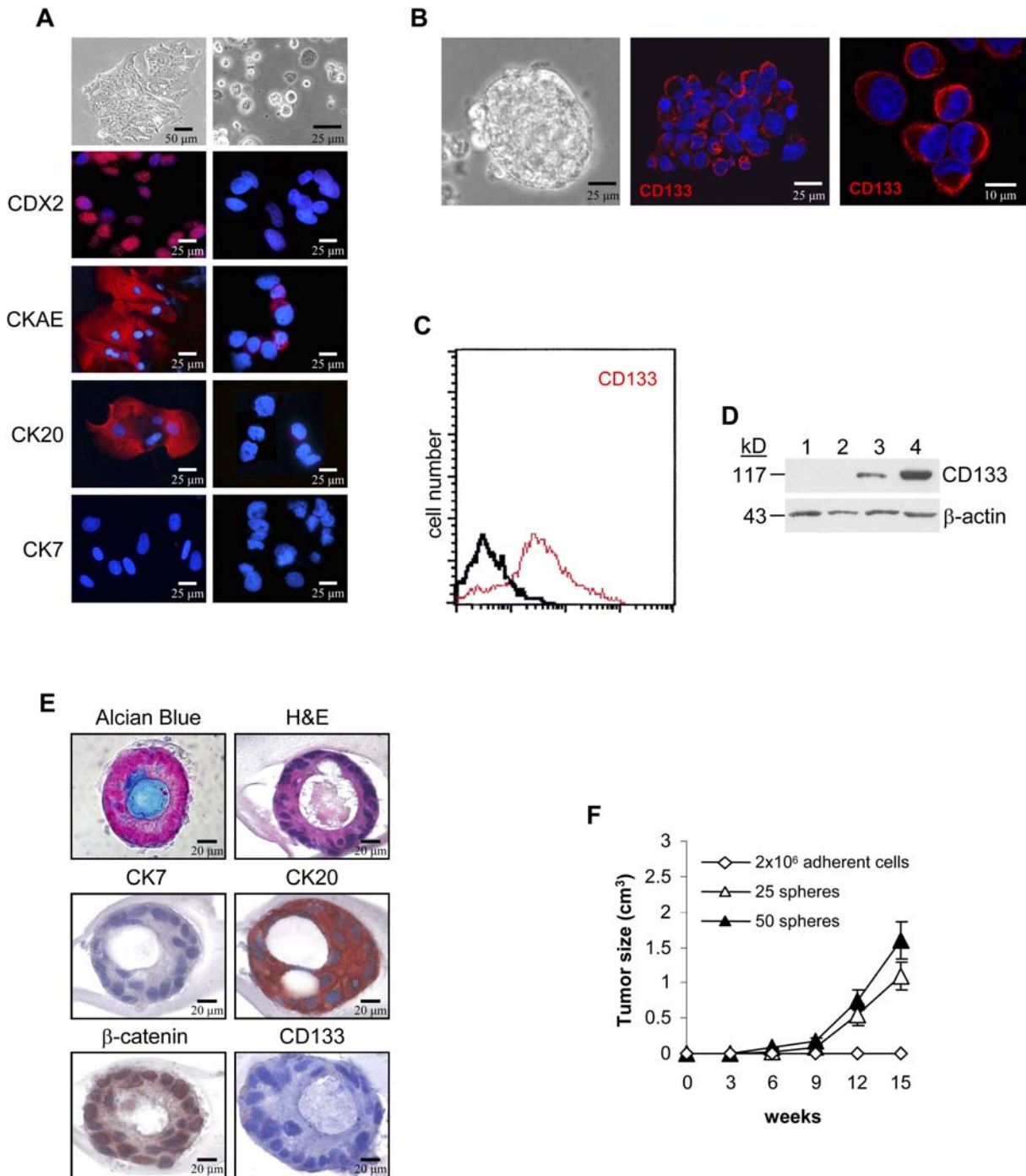


Figure 2. Colorectal Cancer Sphere Formation

(A) Phase contrast microscopy and immunofluorescence analysis of CDX2, CKAE, CK20, and CK7 (red color) on primary adherent colorectal cancer cells (left panels) and mechanically dissociated cancer spheroid cells (right panels). Nuclei were counterstained by Hoechst (blue staining). One representative experiment of five different tumors is shown.

(B) Phase contrast microscopy of a colorectal cancer sphere (left panel). Confocal microscopy analysis of CD133 on spheroids (middle panel) and mechanically dissociated cancer spheroid cells (right panel). Nuclei were counterstained by Hoechst (blue staining). One representative of four different spheroid cultures is shown.

(C) Flow cytometry analysis of CD133 on mechanically dissociated colon cancer spheroid cells. Black histogram represents isotype control. One representative of six independent tumors is shown.

(D) Immunoblot analysis of CD133: (1) control epithelial colon cells (cultured for 5 days), (2) primary adherent colon cancer cells, and (3) colon cancer spheres. Human cord blood CD34⁺ cells (4) were used as positive control for CD133. Loading control was assessed by β -actin staining. One representative of four independent tumors is shown.

Table 2. Tumorigenicity of CD133⁺ and Spheroid-Initiating Colon Cancer Cell

Cancer Source	Patients	Cell Number Injected	Xenograft Formation	Engraftment-Derived Spheres
Bulk	P1-5-7-8-10-13-14-15-17-19-20-21	5 × 10 ⁵	yes	yes
		2 × 10 ⁶	yes	yes
Spheres	P1-5-7-8-10-13-14-19-20-21	25	yes	yes
		50	yes	yes
CD133 ⁺	P1-5-7-8-10-13-15*-19-21	2.5 × 10 ³	yes	yes
		5 × 10 ³	yes	yes
CD133 ⁻	P1-5-7-8-10-13-19-21	2 × 10 ⁶	no	/
Adherent cells	P1-5-7-8-10-13-14-15-17-19-20-21	2 × 10 ⁶	no	/

* This experiment was done using 3.5 × 10³ and 7 × 10³ CD133⁺ cells.

with oxaliplatin, which resulted in a clear reduction in tumor size (Figures 5A and 5B), led to a significant increase in the percentage of CD133⁺ cells (Figure 5E). This means that the CD133⁺ cells have a survival advantage when tumors are treated with oxaliplatin and therefore show a relative increase. On the contrary, cotreatment with anti-IL-4 ablated this selective advantage (Figure 5E). The percentage of CD133⁺ cells in tumor treated with oxaliplatin plus anti-IL-4 is therefore not significantly different from untreated tumors, but the absolute number of CD133⁺ is largely reduced (Figure 5E) as the tumor size is reduced 5-fold. We can therefore conclude that IL-4 protects CD133⁺ stem-like cancer cells from oxaliplatin treatment in vivo and thereby precludes optimal tumor control by chemotherapy.

DISCUSSION

Cancers are heterogeneous populations of cells at multiple differentiation stages that could be the result of acquired mutations and epigenetic differences. Alternatively, differential expression of differentiation markers could be due to aberrant differentiation orchestrated by a stem cell population (Phillips et al., 2006). Although both hypotheses are difficult to proof, our current data show that colon cancer lesions consist of a large fraction of differentiated cancer cells and a small population of cells that express CD133 stem cell marker. From 10 of the 21 tumors studied, spheroid cultures could be derived, and this appears more effective when studying MSI and high-grade tumors. Although this is not a 100% correlation, it suggests that MSI colon carcinomas may contain specific mutations that facilitate their in vitro growth. In contrast to the sphere cultures, the adherent cell populations established from the same tumor were not tumorigenic in mice, while they display epithelial features and

reflect the histological variants from which they were initially derived. Similar observations were also made for colon (Ricci-Vitiani et al., 2007) and other solid tumors, such as brain, melanoma, ovarian, prostate, and breast carcinoma (Bapat et al., 2005; Collins et al., 2005; Dontu et al., 2004; Fang et al., 2005; Singh et al., 2004).

In analogy with stem cells, CSC are suggested to resist death-inducing signals as they are slow cycling and express high levels of drug transporters (Dean et al., 2005). CSC were also suggested to express high levels of antiapoptotic proteins and thus resist apoptotic stimuli (Eramo et al., 2006). Our spheroid cultures confirm these ideas and show that stem-like tumor cells effectively resist oxaliplatin, 5-FU, and TRAIL-induced death cell. Chemotherapy resistance may in part be due to the slow cycling of CSC, but TRAIL sensitivity is not dependent on the cell cycle. We therefore believe that expression of antiapoptotic proteins dictate this resistance. In agreement, sensitization of spheroid cells with anti-IL-4 downregulates expression of three tested antiapoptotic proteins: cFLIP, PED, and Bcl-x_L. Considerable data suggest that some interleukins promote cancer cell survival and expansion by stimulating expression of antiapoptotic genes. For example, IL-3 increases Bcl-x_L levels in myeloid and pro-B cell (Kieslinger et al., 2000). Similarly, IL-10 upregulates Bcl-2 expression in germinal center B cells, while IL-4 increases survival of malignant B cells (Dancescu et al., 1992). Others and we recently demonstrated that carcinoma cells produce IL-4 and IL-10 and that this alters sensitivity to death receptor- and cytotoxic drug-induced cell death. Neutralization of both IL-4 and IL-10 significantly increased the effectiveness of death receptors and antineoplastic drugs on primary cancer cells (Stassi et al., 2003; Todaro et al., 2006). We now extend these observations to colon CSC both in vitro and in vivo. Our data indicate that IL-4-dependent protection is essential for cell death

(E) Alcian blue, H&E stainings, and immunohistochemical analysis of CK7, CK20, β-catenin, and CD133 performed on paraffin-embedded sections of spheroids cultured in matrigel for 20 days. One representative of four independent spheroid cultures is shown.

(F) Size of subcutaneous tumor growth following injection of colon carcinoma cells from adherent culture (diamonds) and sphere cultures (filled and unfilled triangles) derived from the same colon cancer specimen. Data are mean tumor size ± SD of four tumors per group derived from two separate patients.

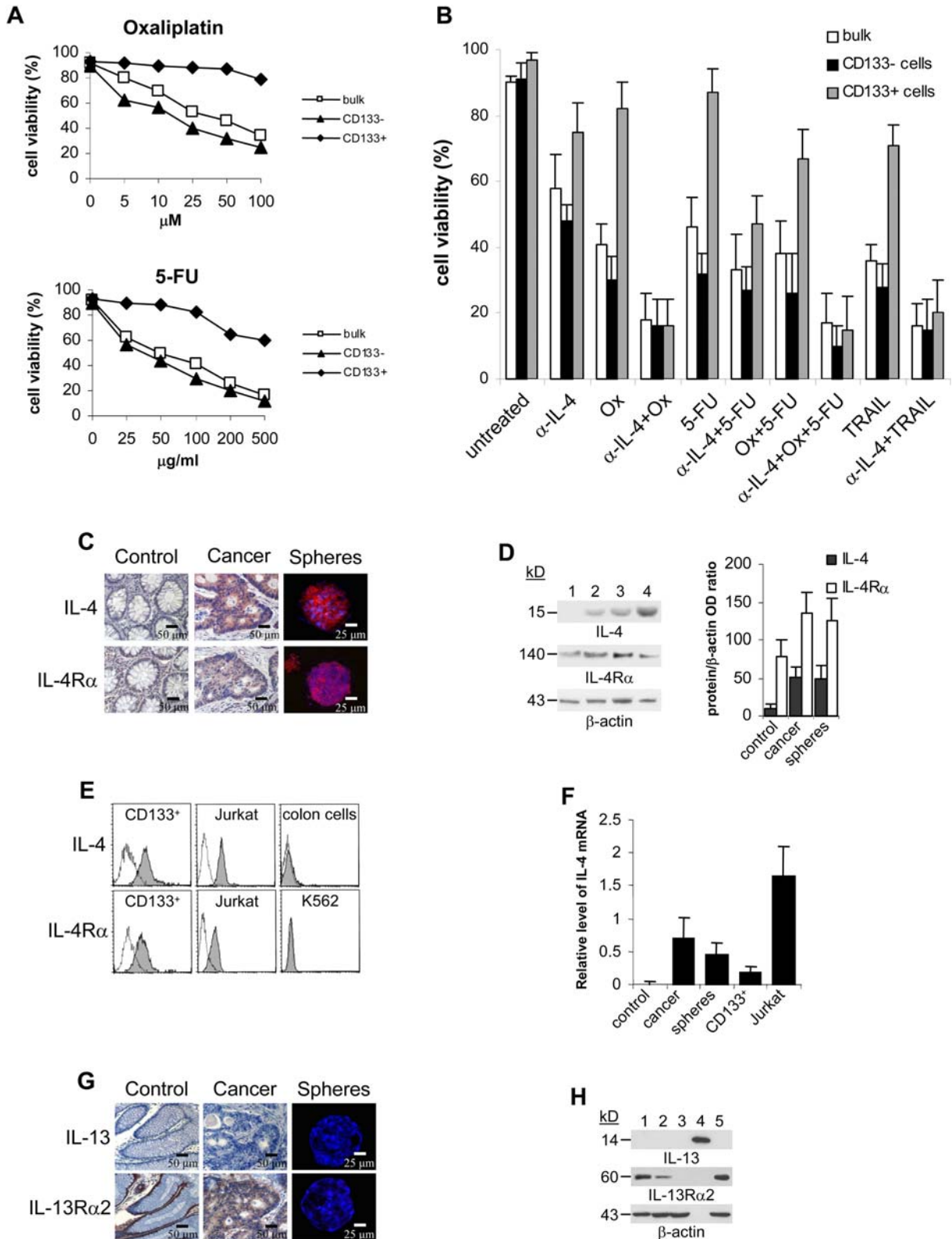


Figure 3. Colon CSC Express and Are Protected by IL-4/IL-4R

(A) Effect of 24 hr exposure of bulk, CD133⁻ and CD133⁺ cells to different concentrations of oxaliplatin or 5-FU.

(B) Percentage of cell viability in freshly obtained colon cancer specimens was analyzed on bulk cells (white bars), on CD133⁺-sorted (black bars), or CD133⁻-sorted (gray bars) cells. Cells were pretreated or not with 10 μg/ml of anti-IL-4 for 24 hr and then cultured with oxaliplatin (100 μM), 5-FU

resistance of colon CSC. Importantly, we observed that standard drug treatment is indeed effective *in vivo* in delaying tumor outgrowth but after cessation of treatment tumor growth is accelerated. This is consistent with the hypothesis that CSC when left unharmed will rapidly regenerate the original tumor. Our findings on IL-4 proof to be a general phenomenon in colon cancer as it was observed in all samples tested (16 out of 18 tested) and not related to stage, location of the tumor, or MSI. These findings may thus provide the means to counteract tumor regeneration by selectively killing CSC.

The IL-4 dependent resistance of tumor cells is likely to be a tumor autocrine event, at least in our experimental conditions. *In vitro*, IL-4-dependent survival of colon cancer cells or CD133⁺ cells was clearly demonstrated to occur as an autocrine event. *In vivo*, a possible paracrine effect of IL-4 can be excluded as well for several reasons. First of all, tumor-derived IL-4 was previously suggested to affect the T cell response, but all our *in vivo* experiments were performed on T cell-deficient mice, excluding an effect of anti-IL-4 on T cell-dependent immunity. Second, IL-4 has long been recognized for its strict species-specific character (Morrison and Leder, 1992), that is, tumor-derived human IL-4 is incapable of acting on the mouse IL-4R α and mouse IL-4 does not influence human (tumor) IL-4R α . The IL-4 related-cytokine IL-13 is not species specific and shares with IL-4 not only the common subunit IL-4R α , but also some common signaling pathways. Thus, we used a specific inhibitor for the IL-4R α subunit to effectively block either cytokine signaling. In addition we also tested an IL-4 neutralizing antibody specific for human IL-4. Both strategies enhanced the tumor response to chemotherapeutic drugs in the same extent, suggesting that the tumor control observed in our murine model was primarily due to impaired IL-4- rather than IL-13-mediated signaling. We can conclude that the *in vivo* effect of tumor-derived IL-4 could solely be propagated via tumor receptors, similar to what we observed in the isolated *in vitro* assays, and it is likely that IL-4 produced by colon cancer cells installs an antiapoptotic program in cancer stem-like cells that protects them from chemotherapy.

Although in our study the immune-system was ruled out to better understand the mechanism underlying survival of tumor stem-like cells, this is not the environment for real epithelial tumors; in fact, many studies in humans reported infiltration of various tumor types with immunoregulatory T cells (Gabrilovich, 2004). In general, inflammatory reactions mediated by Th2 (IL-4 and IL-13) cytokines are known to be the primary cause of several pathologies when they become chronic (Mentink-Kane et al., 2004). In primary prostate, breast, and bladder cancer continuous *in vivo* production of IL-4 was observed, and cancer cells undergo apoptosis only in the absence of this cytokine (Conticello et al., 2004). There is also recent evidence that CD4⁺ T cells infiltrating breast cancer tumors secrete high levels of Th2 cytokines that actually promote cancer cell proliferation, which is counteracted with IL-13 antagonist (Aspord et al., 2007).

Although these studies demonstrate the presence of IL-4 or IL-13 in malignancies, the mechanism that determines which of these cytokines dominate the biological responses and how the components of IL-4/IL-13 receptor complex are regulated are poorly understood. In colon carcinoma there appears to be a primary role for IL-4, as IL-13 is not expressed in the tumor cells, while the IL-13 decoy receptor is. Moreover, anti-IL-4 is as effective as the IL-4DM in blocking the resistance.

The contribution of IL-4 to chemotherapy resistance described in this work contribute to a better understanding of the biological consequences of IL-4 upregulation and the factors that may determine tumor escape from current therapies. Combined, our observations may proof to be of critical importance in future antitumor therapies, as it has been hypothesized that a surviving small population of cancer stem cells could be responsible for tumor reappearance after an apparent complete regression and thus could contribute to minimal residual disease (MRD). The data presented here provide a rational to use anti-IL-4 as a novel approach in antitumor therapies and suggest further investigations into the underlying mechanism of tumor-specific IL-4 expression and resistance.

(50 μ g/ml), oxaliplatin plus 5-FU, or TRAIL (200 ng/ml) for an additional 24 hr. Data are mean \pm SD of six independent experiments, each performed with cells from different donors.

(C) Immunostaining analysis of IL-4 and IL-4R α on paraffin-embedded normal and colorectal carcinoma sections and on colon cancer spheres (red staining). Nuclei were counterstained by hematoxylin or Hoechst (blue color). One representative of 21 tumors for paraffin sections and seven independent experiments for spheres is shown.

(D) Immunoblot analysis of IL-4 and IL-4R α in (1) control epithelial colon cells, (2) primary adherent colon cancer cells or (3) colon cancer spheres and their relative band density. PMA/Ionomycin treated Jurkat cells (4) were used as positive control. Loading control was performed by β -actin staining. Western blot is one representative out of three performed. Graph represents the mean \pm SD of these three independent experiments, each using cells from different patients.

(E) Flow cytometry analysis of IL-4 and IL-4R α on freshly isolated magnetically sorted CD133⁺ cells, PMA/Ionomycin-treated Jurkat cells, control epithelial colon cells (cultured for 5 days), and K562 cells (control = unfilled, stained = filled histogram). One representative of four independent experiments is shown.

(F) mRNA expression of IL-4 in control epithelial colon cells, adherent colon cancer cells, colon cancer spheroid, or freshly isolated CD133⁺ cells. PMA/Ionomycin-treated Jurkat cells were used as positive controls. One representative patient of three is shown.

(G) Immunostaining analysis of IL-13 and IL-13R α 2 as in (C). Representative for 20 tumors (paraffin sections) and four spheroid cultures.

(H) Immunoblot analysis of IL-13 and IL-13R α 2 in (1) control epithelial colon cells, (2) primary adherent colon cancer cells, (3) colon cancer spheres, (4) hIL-13 or (5) human glioblastoma cell line TB10 as positive control. Loading control was performed by β -actin staining. Immunoblot is one representative out of three performed.

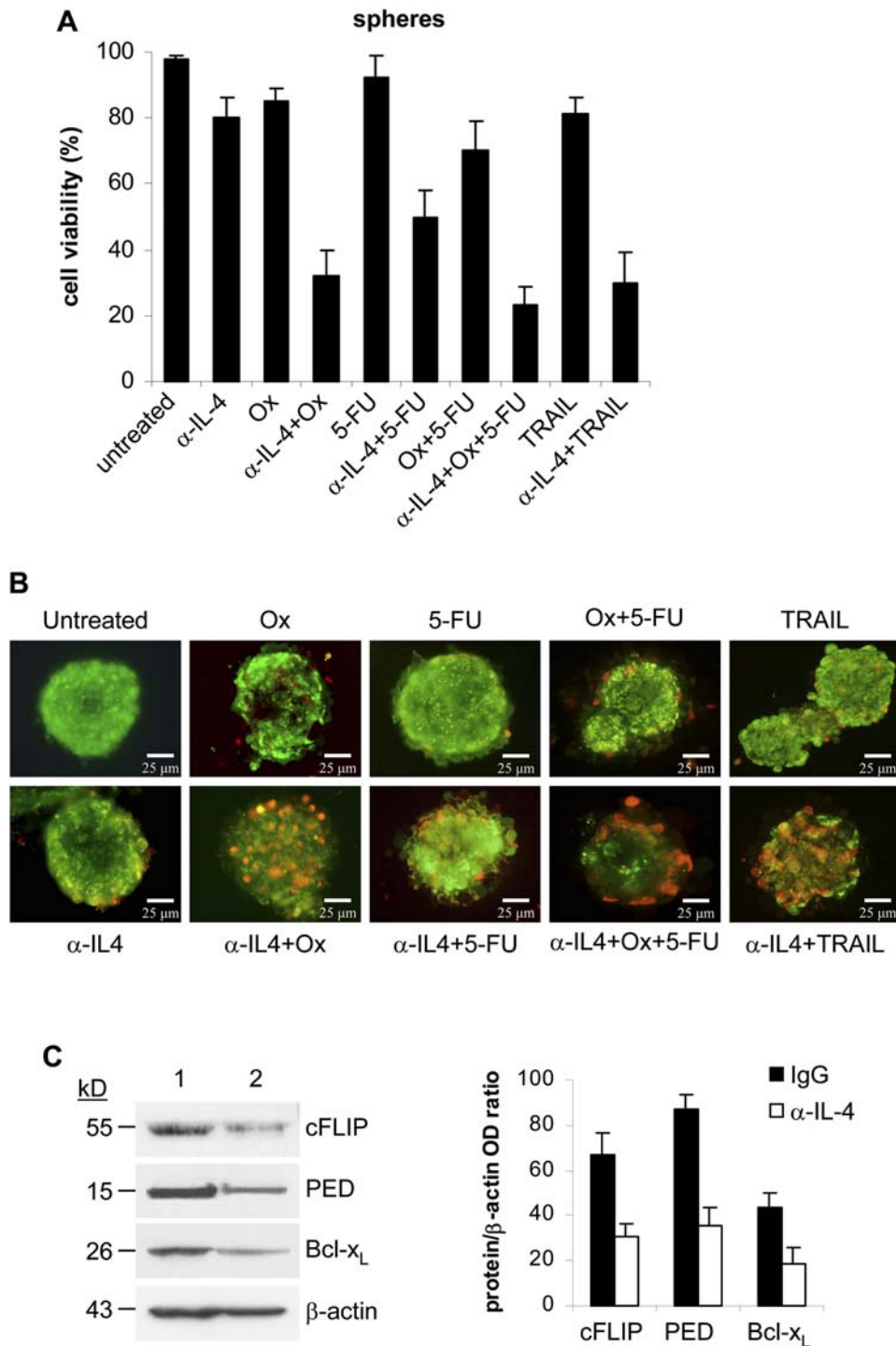


Figure 4. IL-4 Protects Colon Cancer Spheres from Cell Death

(A) Cell viability percentage of colon cancer spheres pretreated for 24 hr or not with 10 μ g/ml of anti-IL-4 and then cultured with oxaliplatin, 5-FU, oxaliplatin plus 5-FU or TRAIL for additional 24 hr. Data represent mean \pm SD of four independent experiments each performed with cells from different donors.

(B) Immunofluorescence analysis of orange acridine/ethidium bromide stained colon cancer spheres treated as in (A). One representative of six independent experiments is shown.

(C) Immunoblot analysis of cFLIP, PED, and Bcl-x_L in colon cancer spheres (1) cultured in the presence of neutralizing antibody against IL-4 for 48 hr (2) and its relative band density of four independent experiments, each using spheres from different patients. Loading control was assessed by β -actin staining.

EXPERIMENTAL PROCEDURES

Tissue Collection, Isolation, and Culture of Cancer Cells

Human colon tissue fragments were obtained in accordance with the ethical standards of the institutional committee on human experimentation from 21 patients (age range 55–70 years) undergoing a colon resection for colon adenocarcinoma. Histological diagnosis was based on microscopic features of carcinoma cells determining the histological type and grade. Cancer tissues were intensively washed four times in PBS solution containing antibiotics and incubated overnight in DMEM/F12 (GIBCO) containing penicillin (500 U/ml), streptomycin (500 µg/ml), and amphotericin B (1.25 µg/ml) (GIBCO). Enzymatic digestion was performed using collagenase (1.5 mg/ml) (GIBCO) and hyaluronidase (20 µg/ml) (Sigma Chemical) in PBS for 1 hr. Then, the digests were used for (1) direct engraftment, (2) depletion or purification of CD133⁺ cells (see below), (3) culturing in DMEM supplemented with 10% fetal bovine serum (FBS), L-glutamine and 1x antibiotic-antimycotic (GIBCO) onto collagen (Calbiochem) coated flasks to obtain primary colon cells, and (4) culturing in stem cell medium DMEM/F12 (GIBCO) supplemented with 6 mg/ml Glucose, 1 mg/ml NaHCO₃, 5 mM HEPES, 2 mM L-Glutamine, 4 µg/ml Heparin, 4 mg/ml BSA, 10 ng/ml bFGF, 20 ng/ml EGF, 100 µg/ml apotrasferrin, 25 µg/ml insulin, 9.6 µg/ml putrescine, 30 nM sodium selenite anhydrous, and 20 nM progesterone (Sigma) to obtain spheroids. To enhance epithelial *in vitro* cytodifferentiation of stem cell-like cells, spheroids were exposed to DMEM supplemented with 1% FBS. All cell culture was carried out at 37°C in a 5% CO₂ humidified incubator.

Control tissue specimens, obtained from the peritumoral tissues of the surgically removed specimens, were digested with collagenase P (0.4 U/ml) and dispase I (1.2 U/ml) for 4 min at 37°C. Cells were obtained by mechanical dissociation of crypts and kept in culture up to 5 days (Fonti et al., 1994).

Magnetic Sorting and Flow Cytometry

Magnetic cell separation was performed on tumor cell populations obtained from enzymatic dissociation of colon cancer specimens using microbeads conjugated with CD133/1 (AC133, mouse IgG₁, cell isolation kit, Miltenyi). The magnetic separation step was repeated twice, applying the eluted cells to a new positive selection column. After magnetic sorting, viability was assessed using trypan blue exclusion. Quality of sorting was controlled by flow cytometry with an antibody against CD133/2 (293C3-PE, Miltenyi) on both CD133⁺ and CD133⁻ depleted cell population. Fractionated and nonfractionated cells from human specimens and xenografts (see below) were analyzed for CD133 expression using CD133/2 (293C3-PE, Miltenyi). IL-4 and IL-4R α expression were assessed on CD133⁺ cells using FITC-conjugated anti-IL-4 (B-S4, mouse IgG₁, Bender), biotin-conjugated anti-CD124 (hIL4R-M57, mouse IgG₁, BD PharMingen), or isotype-matched control (mouse IgG₁ FITC and mouse IgG₁ biotinylated BD PharMingen and rabbit anti-CD3 ϵ Chemicon). Streptavidin-FITC was used for detection of IL-4R α . Cells were washed and analyzed by flow cytometry. Jurkat exposed for 4 hr to PMA (10 ng/ml) and ionomycin (1 µM), control colonocytes cultured for 5 days and K562 cell lines were used as positive and negative controls respectively.

In Vitro Cell Death Analysis

Freshly isolated colon cancer cells were either cultured as nonfractionated or first magnetically sorted into CD133⁺ and CD133⁻ cells and then cultured in DMEM plus 1% FBS. Where indicated, cells were pretreated for 24 hr with anti-human IL-4 (10 µg/ml) (R&D Systems). Similarly, spheroid cultures from the same tumor specimen were cultured in the presence or absence of anti-human IL-4 in stem cell medium. Fresh anti-IL-4 (10 µg/ml) was added after 24 hr and cells were subsequently treated for an additional 24 hr with oxaliplatin (100 µM) (Sigma) or with 5-FU (50 µg/ml) (Sigma) or in combination (oxaliplatin plus 5-FU) or iso-leucine zipper TRAIL (200 ng/ml; iz-TRAIL kindly provided by H. Walczak), after which cell death was determined. Viability of freshly isolated CD133⁺, CD133⁻, and complete pool cells was evalu-

ated using CellTiter Aqueous Assay Kit (Promega). Percentage of cell death on colon cancer spheres was evaluated by DNA staining followed by flow cytometry analysis. Moreover, cell death on colon cancer spheres was evaluated by confocal analysis of orange acridine/ethidium bromide stained cells.

Histochemistry, Immunohistochemistry/Fluorescence, and TUNEL Staining

Histochemical analysis was performed on 5-µm-thick paraffin-embedded sections of colon specimens or in 3D cell culture. H&E- and Alcian blue-stained sections were dehydrated and mounted in synthetic resin. Slides were heated for antigen retrieval in 10 mM sodium citrate (pH 6.0). After rinsing in dH₂O, inhibition of endogenous peroxidase was performed with 3% H₂O₂ (5 min). After two washes in TBS, slides were incubated with 10% human serum for 20 min to block nonspecific staining. Following elimination of excess serum, sections were subsequently exposed to specific antibodies against cytokeratin 7 (OVTL mouse IgG_{1K}, BioGenex), cytokeratin 20 (IT-Ks20.8 mouse IgG_{2a}, BioGenex), Ki67 (MIB-1 mouse IgG₁, Dako), CDX2 (CDX2-88 mouse IgG₁, Biocare), CD133/2 (Miltenyi), β -catenin (17C2 mouse IgG_{2a} Novocasttra), IL-4R α (C-20 rabbit IgG, Santa Cruz), IL-4 (B-S4 mouse IgG₁, Bender MedSystems), IL-13 (B-P6 mouse IgG₁, Bender), IL-13 R α 2 (AF146 goat IgG, R&D Systems), or isotype-matched controls at appropriate dilutions overnight at 4°C. Then sections were washed in TBS and incubated with biotinylated anti-mouse or anti-rabbit immunoglobulins for 30 min at room temperature and treated with streptavidin-peroxidase (LSAB2 Kit, Dako). Staining was revealed using AEC substrate and counter-stained with hematoxylin.

Immunofluorescence was performed on spheroids, on mechanically dissociated spheroid cells, or on primary adherent cultures of colon cancer cells for cytokeratin 20 (BioGenex), CD133/2 (Miltenyi), IL-4R α (Santa Cruz), IL-4 (Caltag), IL-13 (Bender), IL-13 R α 2 (R&D Systems) cytokeratin 7 (BioGenex), cytokeratin A/E (AE1/AE3 mouse IgG₁, Dako), and CDX2 (Biocare Medical) stained sections were treated with Rhodamine Red-conjugated anti-mouse or anti-rabbit antibodies (Molecular Probes, Inc., Eugene, OR). Counterstaining was performed using Hoechst 33342. Confocal analysis was used to acquire fluorescence staining.

TdT-mediated dUTP nick end labeling (TUNEL)-reaction was performed on paraffin-embedded CD133-labeled engrafted tumor sections (10 µm). Specimens were postfixed with paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Apoptosis was determined by In Situ Cell Death Detection, AP Kit (Boehringer Mannheim). DNA strand breaks were detected by 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, dark blue color, Dako) substrate. Positive control was performed by pretreating specimens with DNase I, while the negative control was represented by CD133 positive specimens subjected to the same staining for TUNEL without TdT.

Protein Isolation and Western Blotting

Spheroids and cell pellets were resuspended in ice-cold NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% NP-40) containing protease inhibitors. Lysates (30 µg/lane) were applied to SDS-PAGE. Immunoblotting of Abs specific for CD133/2 (AC141 mouse IgG₁, Miltenyi), IL-4 (MAB304, mouse IgG₁, R&D Systems), IL-4R α (MAB230, mouse IgG_{2a}, R&D Systems), IL-13 (Bender), IL-13 R α 2 (R&D Systems), cFLIP (NF6 mouse IgG₁, Alexis), PED/PEA-15 (rabbit IgG kindly provided by G. Condorelli) and Bcl-x_L (H-5, mouse IgG₁; Santa Cruz), or actin (Ab-1, mouse IgM, Calbiochem) were detected using HRP-conjugated anti-mouse or anti-rabbit Abs (Amersham) and visualized by chemiluminescence detection system (West Dura Substrate, Pierce).

Real-Time PCR

Total RNA was extracted and reverse transcribed. TaqMan PCR reactions were performed with ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using 1x probes and primer sets Hs00174122_m1 (IL-4), Hs00174379_m1 (IL-13) or Hu GAPDH

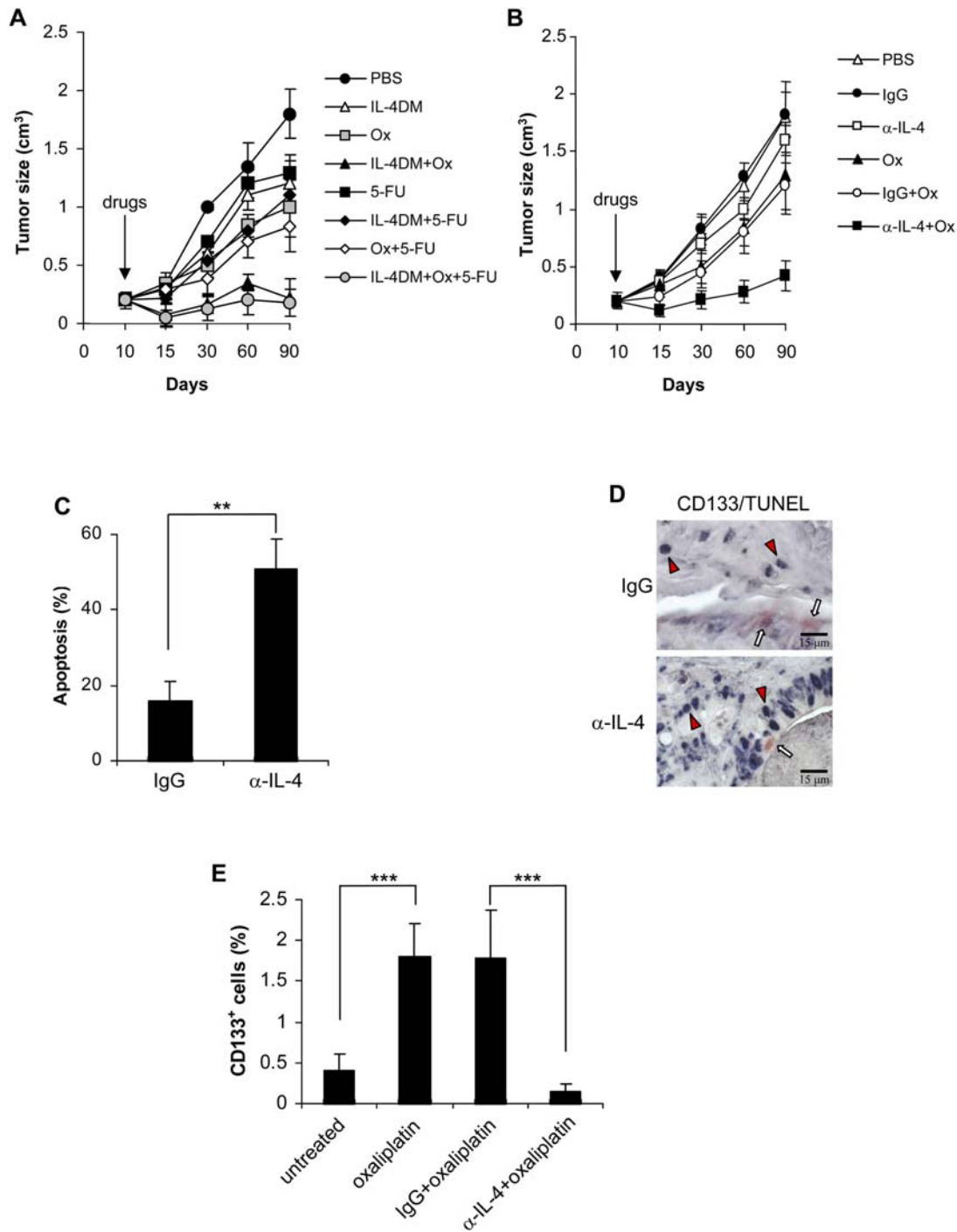


Figure 5. Neutralization of IL-4 Sensitizes Colon Cancer Cells to Cell Death In Vivo

(A) Size of subcutaneous colon carcinoma tumors derived from spheroid injection of a MSS tumor up to 90 days after i.p. treatment with PBS or IL-4DM alone or in combination with oxaliplatin, 5-FU or oxaliplatin plus 5-FU. Data are mean \pm SD of five tumors per group derived from one spheroid culture.

(B) Tumor size derived as in (A) after intratumoral injection of anti-IL-4 or control IgG alone or in combination with i.p. administration of oxaliplatin or PBS. Data are mean tumor size \pm SD of 12 tumors per group derived from two independent experiments using spheroid cultures from different MSI patients.

(C) Percentage of apoptotic events measured on sections of subcutaneous tumors (4 per group) treated as in (B) and stained for TUNEL. $**p < 0.01$. 5×10^5 cells per tumor were counted and the percentage of apoptotic cells calculated. Samples were analyzed 6 weeks after treatment started.

(D) CD133 (red color, white arrows) and TUNEL (dark blue color, red arrowheads) double staining of tumors treated as in (B).

(Applied Biosystems). The reaction conditions were: 95°C for 10 min and 50 cycles at 95°C for 15 s and 60°C for 1 min. The relative quantitation of IL-4 and IL-13 gene expression was calculated using the comparative Ct method ($\Delta\Delta Ct$). Experimental target quantities were normalized to the endogenous Hu GAPDH control. Data processing and statistical analysis were performed using the ABI PRISM SDS, software version 2.1 (Applied Biosystems). Peripheral blood mononuclear cells (PBMC) treated for 4 hr with lipopolysaccharide (LPS) (10 $\mu\text{g}/\text{ml}$) were used as positive control for IL-13.

Evaluation of Tumorigenicity and Toxicity

Tumorigenicity was determined by s.c. injecting either freshly isolated enzymatic dissociated tumor cells (Bulk; 0.5 or 2×10^6), freshly isolated CD133-depleted tumor cells (2×10^6), freshly isolated CD133⁺ cells (2.5 or 5×10^3), adherently growing colon cancer cells (2×10^6), colon cancer spheres (25, 50, and 500), or dissociated spheroids cells (5×10^2) into the flank of 5- to 6-week-old female nude mice. Mice were purchased from Charles River Laboratories (Milan, Italy) and maintained in accordance to the institutional guidelines of the University of Palermo animal care committee. After 10 days, when well-established tumors of about 0.25 cm^3 were detected, 12 mice per group were i.p. treated with a specific inhibitor of IL-4R α , IL-4DM (3 mg/Kg/day twice a week, for 3 weeks) which has substitution at positions 121 (Arg \rightarrow Asp) and 124 (Tyr \rightarrow Asp) (kindly provided by Apogenix) (Zurawski et al., 1993) alone or in combination with oxaliplatin (0.25 mg/kg once a week for 4 weeks) and/or with 5-FU (15 mg/kg/day for 5 days a week for 2 weeks). Additional treatments with neutralizing Abs against IL-4 (R&D Systems; 10 $\mu\text{g}/\text{cm}^3$ twice a week for 3 weeks) administered into the tumor alone or in combination with oxaliplatin (i.p.) were performed in another group of 12 mice. Controls were carried out using IgG isotype matched control antibody or PBS (untreated). Tumor size was measured twice weekly using the formula: $(\pi/6) \times \text{larger diameter} \times (\text{smaller diameter})^2$. Incidence of cell death and CD133⁺ cells was determined 6 weeks after spheroid injection using AC133 and TUNEL staining on a minimum of 10 sections per tumor and a total of around 5×10^5 tumor cells per sample. Counting was performed blinded and by two independent observers on four samples per group derived from two independent spheroid cultures (different patients).

Statistical Analysis

Percentage of apoptotic events was calculated from the number of cells stained for TUNEL on paraffin-embedded sections of subcutaneous tumors. 5×10^5 cells were counted per tumor ($n = 4$).

For in vitro cell death quantification, percentage of cell death was derived from the percentage of viable cells that was calculated by MTS assay or the Propidium Iodide staining. CD133⁺ cells percentage was calculated from the positivity for CD133 observed on paraffin-embedded engrafted tumor tissues counted by two independent observers or flow cytometry analysis assessed on engrafted-derived complete pool cells. Analysis of Variance (one-way or two-way) with Bonferroni post-test was used to analyze the statistical significance of the experimental results. Results were considered significant when p values were less than 0.05.

Intensity of band signals in exposed film was determined by densitometric scanning and analyzed using NIH IMAGE software version 1.62 (by Wayne Rasband, National Institutes of Health, Research Services Branch, NIMH). Results were expressed as protein/ β -actin OD ratio (OD). Data were expressed as mean \pm standard deviation of the mean.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, two supplemental figures, and two supplemental tables and

can be found with this article online at <http://www.cellstemcell.com/cgi/content/full/1/4/389/DC1/>.

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(E) Percentage of CD133⁺ cells evaluated on paraffin-embedded sections stained for CD133 of tumors untreated and treated with oxaliplatin alone or in combination with IgG or anti-IL-4. Data are mean \pm SD of four tumors per group and derived by counting a minimum of 10 different sections and around 5×10^5 cells per tumor. Tumors were derived from two independent experiments with spheroid cells from different donors. *** $p < 0.001$ versus untreated or IgG plus oxaliplatin.

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