

Apoptosis resistance in epithelial tumors is mediated by tumor-cell-derived interleukin-4

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We investigated the mechanisms involved in the resistance to cell death observed in epithelial cancers. Here, we identify that primary epithelial cancer cells from colon, breast and lung carcinomas express high levels of the antiapoptotic proteins PED, cFLIP, Bcl-xL and Bcl-2. These cancer cells produced interleukin-4 (IL-4), which amplified the expression levels of these antiapoptotic proteins and prevented cell death induced upon exposure to TRAIL or other drug agents. IL-4 blockade resulted in a significant decrease in the growth rate of epithelial cancer cells and sensitized them, both *in vitro* and *in vivo*, to apoptosis induction by TRAIL and chemotherapy via downregulation of the antiapoptotic factors PED, cFLIP, Bcl-xL and Bcl-2. Furthermore, we provide evidence that exogenous IL-4 was able to upregulate the expression levels of these antiapoptotic proteins and potently stabilized the growth of normal epithelial cells rendering them apoptosis resistant. In conclusion, IL-4 acts as an autocrine survival factor in epithelial cells. Our results indicate that inhibition of IL-4/IL-4R signaling may serve as a novel treatment for epithelial cancers.

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Resistance to induction of cell death in cancer cells represents one of the major obstacles to successful cancer treatment. Multiple mechanisms of drug resistance in tumor cells have been identified, including decreased cellular uptake or increased cellular efflux of chemotherapeutic drugs as well as alterations in signaling pathways controlling the cell cycle or induction of cell death by apoptosis.¹ Combination of different conventional therapies, including chemotherapy, radiation and surgery, so far have had only a limited success regarding disease-free and/or overall survival.

One of the hallmarks of cancer cells implicated in tumor development as well as metastasis formation is the resistance to apoptosis induction by both death ligands and chemotherapeutic drugs, which trigger the 'extrinsic' and the 'intrinsic' apoptotic pathways, respectively.^{1–3} The death ligand TRAIL (Apo2L) has been shown to specifically induce apoptosis in many tumor cell lines while sparing most normal cells.⁴ Similar to CD95 (Fas/APO-1), binding of TRAIL receptor 1 (TRAIL-R1/DR4) and TRAIL-R2 (Apo2, DR5, KILLER and TRICK2) by its ligand induces receptor clustering and formation of the death-inducing signaling complex (DISC).⁵ Activation of caspases 8 and 10 at the TRAIL DISC can be inhibited by recruitment of the caspase 8/10-homologous proteins cFLIP or PED/PEA-15.^{6–8} By contrast, antineoplastic drugs are a class of cytotoxic compounds that primarily activate the intrinsic apoptosis pathway,

mostly via induction or activation of the BH3-only members of the Bcl-2 family, which results in the release of death factors, such as cytochrome *c* and Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI) from mitochondria.⁹ Both pathways converge in the activation of a caspase cascade. Interestingly, epithelial cancer cells, which are resistant to death receptor-induced apoptosis, are often found to be co-resistant to apoptosis induction by chemotherapeutic drugs and radiation.^{10,11}

Several cytokines have been found to be secreted in the tumor microenvironment by cancer or infiltrating T cells.¹² We had previously found that in Graves' disease, infiltrating T helper type 2 (T_H2) cells produce interleukin-4 (IL-4) and interleukin-10 (IL-10), which make thyrocytes refractory to the apoptosis-inducing effects of the CD95 ligand (CD95L/FasL).¹³ We next examined the thyroid cancer microenvironment for a possible presence of these two cytokines and surprisingly found that the tumor cells themselves, rather than infiltrating T cells, produced both IL-4 and IL-10. IL-4 and IL-10 are pleiotropic cytokines with important physiological functions in the regulation of the immune response. IL-4 plays a central role in regulating the differentiation of antigen-stimulated naive T cells as it determines these cells to develop into IL-4-producing T_H2 cells, which can then also produce IL-5, IL-10 and IL-13.¹⁴ In addition, T_H2 cytokines

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Abbreviations: IL-4, interleukin-4; IL-10, interleukin-10; T_H2, T helper type 2; Abs, antibodies; anti-IL-4, interleukin-4-neutralizing monoclonal antibody

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downregulate antitumor immunity leading to cancer progression and metastasis formation.¹⁵

We found that IL-4 and IL-10 induced growth and resistance to chemotherapy and death ligand-induced apoptosis in thyroid carcinomas. Interestingly, only the concomitant inhibition of IL-4 and IL-10 resulted in sensitization to apoptosis by chemotherapy and death ligands.^{16,17} Here, we studied whether the production of IL-4 and/or IL-10 and the consequent modulation of apoptosis-related proteins, conferring refractoriness to death ligand- and drug-induced apoptosis, may be a mechanism involved in cell survival, tumor progression and therapy resistance in other epithelial cancer cells.

Results

Epithelial cancer cells are resistant to apoptosis induction. To determine the apoptosis sensitivity of primary human epithelial cancer cells to chemotherapy and TRAIL, we isolated primary cancer cells from 20 colon, 10 breast and 4 lung, and cultivated them in the presence or absence of either chemotherapeutics usually used to treat these tumor types or with a recombinant isoleucine-zipper-tagged form of human TRAIL (iz-TRAIL). The applied concentration of each chemotherapeutic drug and of TRAIL was determined by time-course and dose-response studies (Figure 1a). In line with the modest efficacy reported in clinical trials, we found that primary epithelial cancer cells of

all the examined histotypes only exhibited moderate sensitivity to both chemotherapy and TRAIL even at times and doses higher than those used in the clinical setting (Figure 1a).

With respect to TRAIL, this result is in contrast to the expectations from studies with cancer cell lines but it is indeed in line with the rather modest efficacy reported from recent clinical trials with various TRAIL receptor agonists. This resistance was even maintained when TRAIL or the chemotherapeutic agents were used at very high concentrations or for extended times (Figure 1a). Of note, the degree of resistance to cell death induction by apoptosis in the different tumor histotypes was quite uniform within the cohorts studied (Figure 1b and c).

Epithelial cancer cells express high levels of antiapoptotic proteins.

To determine the mechanisms responsible for TRAIL refractoriness, we first investigated the presence of the two death-inducing TRAIL receptors, TRAIL-R1 and TRAIL-R2, showing that both were abundantly expressed on the surface of colon, breast and lung cancer cells (Figure 2a). We thus examined whether aberrant expression of pro- and antiapoptotic factors could be implicated in the observed impairment of the extrinsic and/or intrinsic apoptosis signaling pathway.

We therefore compared the expression of key intracellular regulators of apoptosis in normal *versus* cancer cells from colon, breast and lung, and we found that PED/PEA-15 and

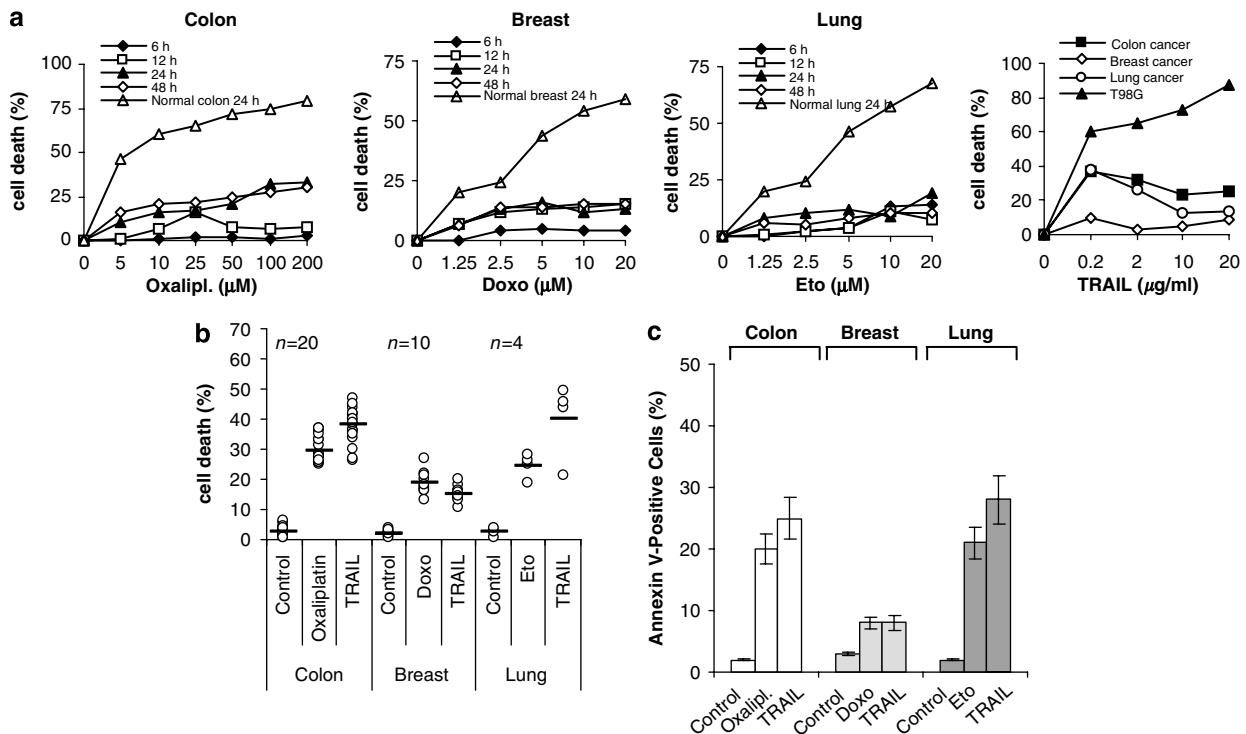


Figure 1 Cell death induction by chemotherapy and TRAIL in epithelial cancer cells. (a) Dose-response curves of epithelial cancer cells for oxaliplatin (oxalip; 0–200 μM), doxorubicin (doxo; 0–20 μM) and etoposide (eto; 0–20 μM) up to 48 h treatment and for TRAIL (0–20 μg/ml) after 12 h treatment. (b) Cell death of colon, breast and lung cancer cells in the presence or absence of oxaliplatin (100 μM), doxorubicin (doxo; 5 μM), etoposide (eto; 5 μM) for 24 h or TRAIL (200 ng/ml) for 12 h. Each point represents data from an individual patient. Data are mean ± S.D. of 20 independent experiments for colon, 10 for breast and 4 for lung. (c) Percentage of annexin V-positive cells assessed by cytometry on colon, breast and lung epithelial cancer cells treated as in (b)

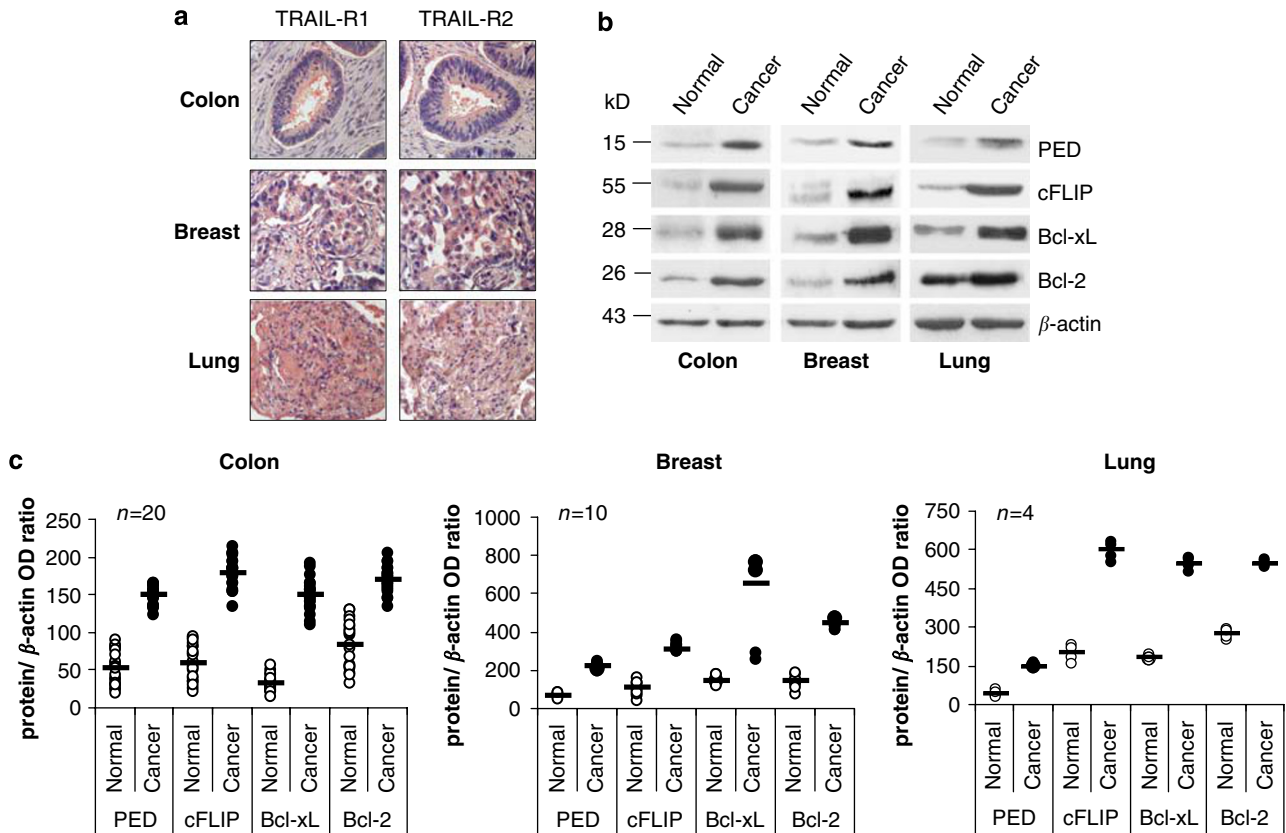


Figure 2 Expression of TRAIL receptors and various apoptotic proteins in epithelial cancer cells. (a) Immunohistochemical analysis of TRAIL-R1 and TRAIL-R2 on paraffin-embedded colon, breast and lung cancer sections revealed by 3-amino-9-ethylcarbazole (red staining). (b) Immunoblot analysis of PED, cFLIP, Bcl-xL and Bcl-2 in freshly purified primary colon, breast and lung normal or cancer cells. Loading control was assessed by detecting β -actin in the same membranes. (c) Relative band densities of antiapoptotic proteins as in (b). Data are representative of 20 independent experiments for colon, 1 of 10 for breast and 1 of 4 for lung

cFLIP levels were approximately three-fold higher in freshly purified cancer cells than in normal cells and that Bcl-xL was increased by about four-fold and Bcl-2 by about two-fold (Figure 2b and c). Thus, the expression levels of various pro-survival proteins, together capable of blocking both arms of the apoptosis signaling pathway, were clearly increased in epithelial cancer cells. These results suggest that the observed resistance to chemotherapy and death receptor triggering could be due to the upregulation of antiapoptotic molecules.

Autocrine production of IL-4 increases growth and survival of epithelial cancer cells. We next investigated whether IL-4 and/or IL-10 and their respective receptors are also present in other types of epithelial neoplasia. Strikingly, immunohistochemical analysis revealed that the vast majority of solid tumors investigated highly expressed IL-4. In summary, tumor cells isolated from 73 of 85 colon (86%), 27 of 32 breast (84%) and 6 of 9 lung (67%) cancers expressed IL-4 (Figure 3a). Moreover, IL-4R α was expressed in all normal and cancer tissues analyzed (Figure 3a). By contrast, we found that IL-10 was either not expressed at all or only at low levels in these tumors (data not shown). This result suggested that neoplastic cells themselves might produce IL-4 that could act in an autocrine/paracrine manner. However, to test whether T-cell infiltrates, possibly

IL-4 producing T_H2 cells, which are often found in tumors, could be the source of this cytokine, we examined whether the reactivity observed in tumor tissues was due to the release of the cytokine by infiltrating T_H2 cells or by the tumor cells themselves. We therefore analyzed freshly purified colon, breast and lung normal and cancer cells on the mRNA and protein levels by real-time PCR and enzyme-linked immunosorbent assay (ELISA), respectively. We controlled for the absence of contaminating infiltrating immune cells by flow cytometry analysis for CD45 (data not shown). In agreement with the results obtained by immunohistochemical analysis, purified cancer cells but not primary cells obtained from normal related tissue specimens expressed IL-4 at both mRNA (Figure 3b) and protein levels, the latter was detectable in colon, breast and lung cancer cells at the levels of 4.6 ± 1.2 , 5 ± 1.8 and 3.8 ± 1.8 pg/ml, respectively (Figure 3c). This result demonstrates that the autocrine production of IL-4 apart from thyroid tumors applies also to other malignant epithelial tumors.

IL-4R α was also expressed in normal cells. Hence, tumor-derived IL-4 may not only affect cancer cells but also the tumor microenvironment by influencing the growth behavior of surrounding normal cells. Thus, to investigate a possible role of IL-4 on normal cells, we cultured freshly purified epithelial cells from colon, breast or lung in the presence or absence of IL-4 and determined cell growth. We found that IL-4

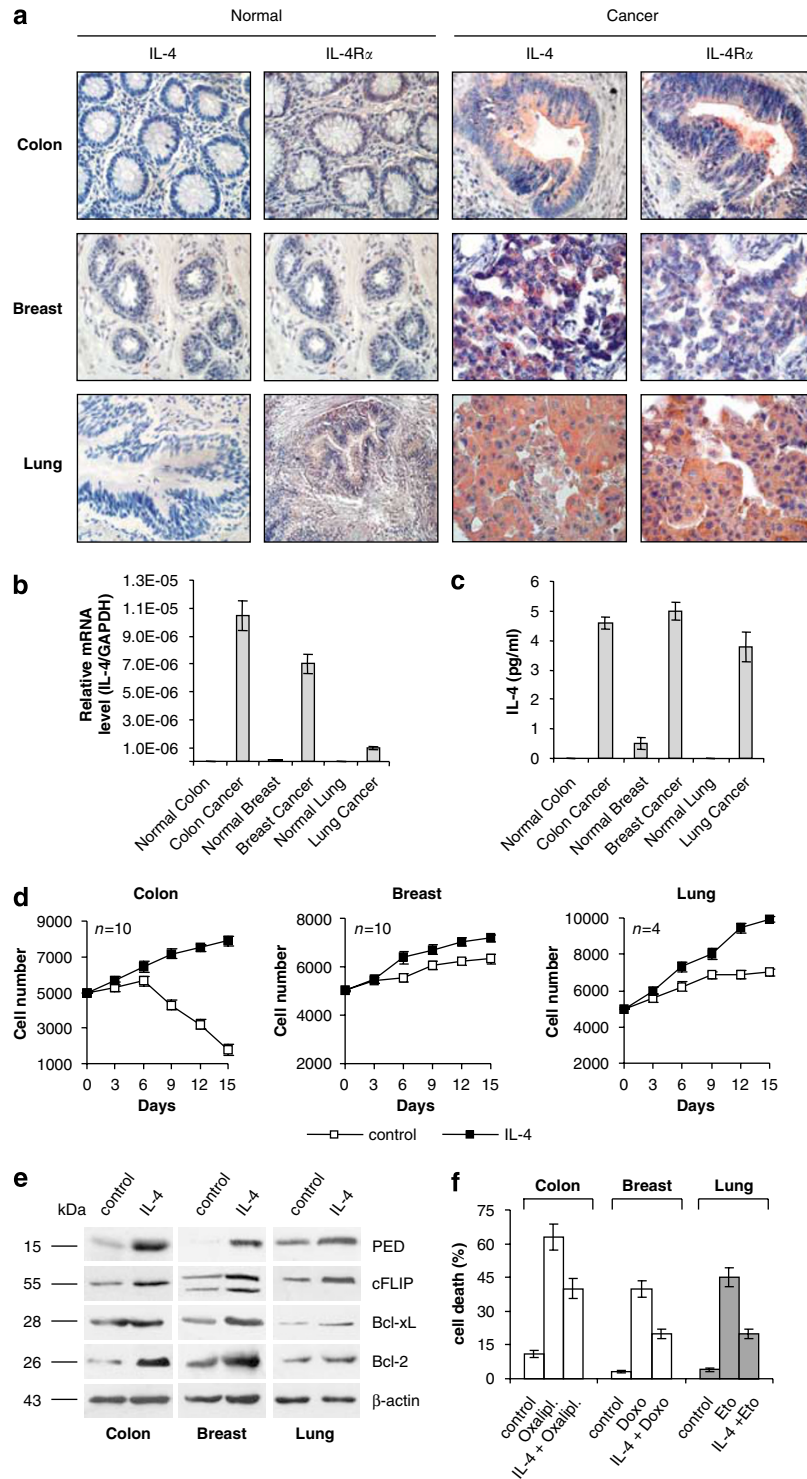


Figure 3 IL-4 is autocrinely produced by epithelial cancer cells and increases survival rate. **(a)** Immunohistochemical analysis of IL-4 and IL-4R α on paraffin-embedded sections from colon, breast and lung normal or cancer specimens (red staining). One representative of 73 of 85 colon, 27 of 32 breast and 6 of 9 lung cancer specimens is shown. **(b)** Real-time PCR analysis of IL-4 mRNA levels on freshly purified normal and cancer cells from colon, breast and lung tissues. **(c)** Quantification of IL-4 production in freshly purified normal and cancer cells as in **(b)** by an IL-4-specific ELISA. **(d)** Cell growth of colon, breast and lung primary normal cells up to 15 days following exposure to IL-4 (20 ng/ml) (filled squares) or PBS (open squares). **(e)** Western blot analysis of PED, cFLIP, Bcl-xL and Bcl-2 in purified colon, breast and lung normal cells following exposure to IL-4 for 48 h. Loading control was performed by using β -actin in the same membranes. Data are representative of 10 independent experiments for colon and breast and 4 for lung, each using specimens or cells from different donors. **(f)** Cell death percentage of primary normal cells untreated or pretreated with IL-4 (20 ng/ml) for 72 h and subsequently exposed to oxaliplatin (oxalipl; 100 μ M), doxorubicin (doxo; 5 μ M) or etoposide (eto; 5 μ M) for 24 h. The results shown are the mean \pm S.D. of four independent experiments

significantly increased the growth rate of primary cells isolated from all these tissues (Figure 3d). Moreover, exogenous IL-4 increased the protein levels of PED/PEA-15, cFLIP, Bcl-xL and Bcl-2 in normal colon, breast and lung cells (Figure 3e) and rendered not only cancer cells but also surrounding non-transformed cells less sensitive to chemotherapy-induced apoptosis (Figure 3f).

IL-4 neutralization promotes growth arrest and cell death induced by TRAIL or chemotherapy in cancer cells. To test whether the autocrine production of IL-4 was responsible for resistance to chemotherapy- and death ligand-induced cell death and possibly also for accelerated growth of the epithelial cancer cells, we next investigated whether the blockade of IL-4 was capable of reversing this phenotype. We therefore exposed primary colon, breast and lung cancer cells to IL-4-neutralizing monoclonal antibody (anti-IL-4), either alone or in combination with chemotherapeutic drugs, which are usually applied in these types of cancer or TRAIL.

We found that IL-4 neutralization alone already significantly decreased the growth rate of primary colon, breast and lung tumor cells *in vitro* (Figure 4a). Furthermore, we determined the expression levels of the antiapoptotic proteins that were shown to be upregulated in IL-4-treated normal cells. Neutralization of IL-4 was sufficient to efficiently downregulate the expression levels of the antiapoptotic proteins PED/PEA-15, cFLIP, Bcl-xL and Bcl-2 (Figure 4b). To study whether the production of IL-4 by tumor cells could be responsible for apoptosis resistance in epithelial cancer, we exposed freshly purified colon, breast and lung cancer cells to anti-IL-4 either

alone or together with conventional chemotherapeutic agents or TRAIL. We found that in most cases pretreatment with anti-IL-4 strongly sensitized primary epithelial cancer cells to apoptosis induction by conventional chemotherapeutics. Primary colon and lung cancer cells were also sensitized to TRAIL-induced apoptosis, whereas in primary breast cancer cells TRAIL sensitization was only marginal (Figure 5a–d). Notably, some tumor samples retained co-resistance to both combinatorial treatments as observed in 4 of 20 colon, 2 of 10 breast and 1 of 4 lung specimens (Figure 5d).

IL-4 blockade sensitizes cancer cells to chemotherapy or TRAIL *in vivo*. We next determined whether IL-4 may also act as an autocrine survival factor of cancer cells *in vivo*, and whether its neutralization may enhance the therapeutic benefit of systemic treatment with chemotherapy or TRAIL. Considering that adherent primary colon and breast cancer cells fail to engraft in immunodeficient mice (data not shown), we tested tumorigenic human colon T84 and breast BT549 cancer cell lines for the expression of IL-4 and IL-4R α to establish an *in vivo* model. T84 and BT549 cancer cells expressed both IL-4 and IL-4R α (Figure 6a and b). IL-4 production as determined by ELISA was 12 ± 1.2 pg/ml for T84 and 10 ± 0.9 pg/ml for BT549 cancer cell lines (Figure 6b). In addition, IL-4-neutralization in T84 and BT549 resulted in a reduction of cellular growth rate up to 15 days and reduced the levels of the antiapoptotic proteins (Figure 6c and d). Moreover, in the absence of IL-4-neutralization, T84 and BT549 cells were almost completely resistant to *in vitro* cell death induction by oxaliplatin and TRAIL, respectively. However, when cultured in the presence

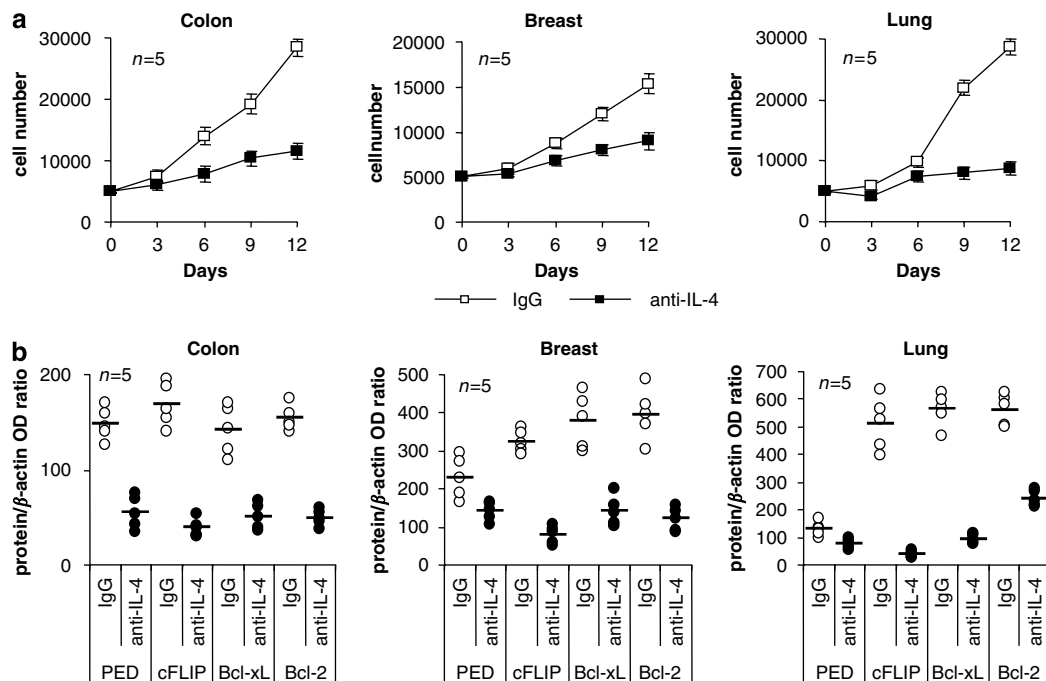


Figure 4 IL-4 neutralization promotes growth arrest in colon, breast and lung carcinoma cells *in vitro*. (a) Cell number of colon, breast and lung cancer cells treated with IgG (open squares) or anti-IL-4 (10 μ g/ml) (filled squares) up to 12 days. (b) Relative band densities of PED, FLIP, Bcl-xL and Bcl-2 in cancer cells treated as in (a). Data are mean \pm S.D. of five independent experiments, each performed with cells from different donors

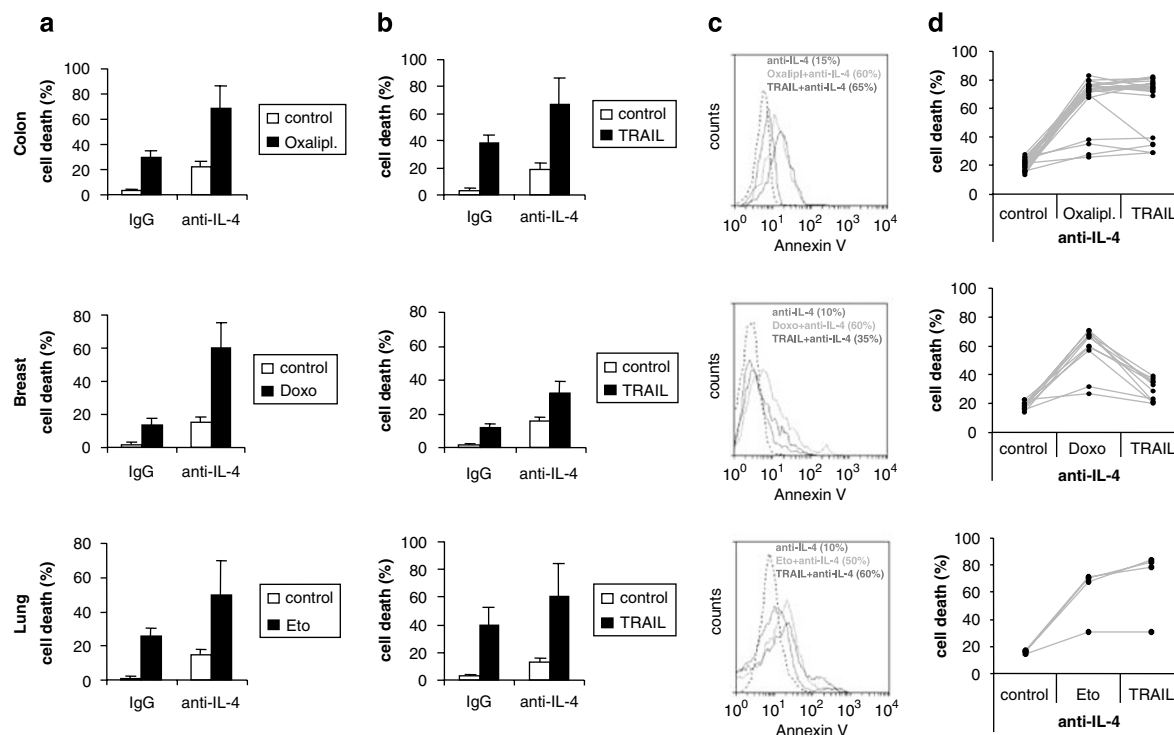


Figure 5 IL-4 neutralization sensitizes colon, breast and lung carcinoma cells to chemotherapy- and TRAIL-induced cell death. (a, b) Cell death percentage of purified colon, breast and lung tumor cells pretreated with IgG or anti-IL-4 (10 µg/ml) for 48 h and subsequently exposed to oxaliplatin (oxalipl.; 100 µM), doxorubicin (doxo; 5 µM) or etoposide (eto; 5 µM), respectively, for additional 24 h (a) or TRAIL (200 ng/ml) (b), for additional 12 h (filled bars). PBS was used as control treatment (open bars). (c) Annexin V representative profiles of colon, breast and lung cancer cells treated as in (a, b). (d) Cell death percentage of purified colon, breast and lung tumor cells treated as in (a, b). Each line represents data from an individual patient. Data are mean ± S.D. of 20 independent experiments for colon, 10 for breast and 4 for lung

of anti-IL-4, both the cell lines were readily sensitized to apoptosis induction by these stimuli (Figure 6e). Yet, to serve as suitable models to test a possible therapeutic effect of inhibition of IL-4 *in vivo*, tumor cells would have to maintain the expression of both IL-4 and its receptor after xenotransplantation. We therefore tested whether this was the case and, indeed we found both proteins to be expressed in subcutaneously grown tumors derived from T84 colon and BT549 breast cancer cells (Figure 7a). Hence, we could next determine whether the neutralization of IL-4 may be of therapeutic benefit *in vivo*. We therefore treated mice carrying either human T84 colon or BT549 breast cancer xenografts with anti-IL-4 alone or in combination with oxaliplatin or TRAIL, respectively. When mice bearing T84 or BT549 tumors were treated with anti-IL-4 alone, tumor growth was only marginally diminished (Figure 7b and c). When mice were treated with oxaliplatin or TRAIL alone, tumor growth was slowed down in both cases. However, this effect was not very pronounced. Taken together, these data indicate that treatment with anti-IL-4, oxaliplatin or TRAIL alone is not sufficient to effectively prevent subcutaneous growth of T84 colon and BT549 breast cancer xenografts (Figure 7b and c). However, when anti-IL-4 was combined with oxaliplatin to treat T84-tumor-bearing mice or with TRAIL to treat BT549-tumor-bearing mice, tumor growth was drastically reduced (Figure 7b and c). *In situ* terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) analysis of paraffin-embedded

sections of T84- and BT459-derived tumor mass in mice treated with anti-IL-4, oxaliplatin or TRAIL alone or in combinations showed that apoptotic events were significantly increased in tumors obtained from mice treated with anti-IL-4 plus oxaliplatin or anti-IL-4 plus TRAIL, respectively (Figure 7d and e). These data indicate that IL-4 neutralization significantly enhances the effectiveness of both chemotherapy and TRAIL treatment through apoptosis induction in tumor cells *in vivo*. Taken together, our findings suggest that the production of IL-4 may play a general role in cancer cell survival and that the interference with its effect in tumor cells may represent a novel strategy for the treatment of epithelial cancers.

Discussion

Apoptosis resistance of cancer cells can be achieved by overexpression of an oncogene or loss of expression of a tumor suppressor gene.¹⁸ Production of immunosuppressive cytokines constitutes an additional survival strategy used by tumor cells to evade the recognition of the immune system. Both infiltrating T cells in the tumor microenvironment and cancer cells can be the source of these molecules.^{12,19,20} It has been shown that T_H2 cytokines downregulate antitumor immunity leading to cancer progression and metastasis formation. Accordingly, patients affected by a variety of human tumors express high levels of T_H2 cytokines in the periphery and in the tumor microenvironment, which correlate

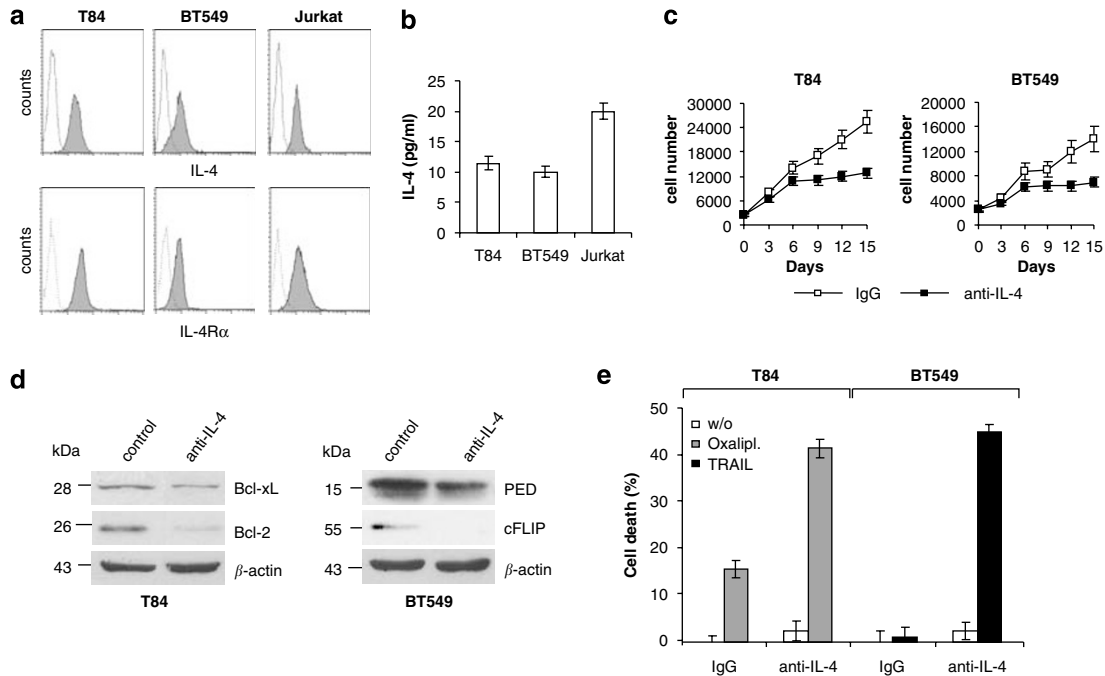


Figure 6 *In vitro* sensitization of colon T84 and breast cancer BT549 cell lines to chemotherapy- or TRAIL-induced cell death following anti-IL-4 treatment. (a) Flow cytometry analysis of IL-4 and IL-4R α on T84 and BT549 cells. PMA/ionomycin-treated Jurkat cells were used as a positive control. (b) Quantification of IL-4 production in T84 and BT549 cells by an IL-4-specific ELISA. (c) Cell number of T84 and BT549 cells after treatment with IgG (open squares) or anti-IL-4 (10 μ g/ml) (filled squares) up to 15 days. (d) Immunoblot analysis of PED, cFLIP, Bcl-xL and Bcl-2 in T84 and BT549 cells treated as in (c). Loading controls were performed by using β -actin in the same membranes. (e) Percentage of cell death on T84 and BT549 cells treated with IgG or anti-IL-4 (10 μ g/ml), either alone (open bars) or in combination with oxaliplatin (100 μ M) (gray bars) or TRAIL (200 ng/ml) (filled bars). Data are mean \pm S.D. of six independent experiments

with the stage and grade of malignancy.^{21,22} Particularly, IL-4 plays a major role in inducing T_H2 responses acting through IL-4R α and activating the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling cascade.^{17,23}

Recently, IL-4 has been shown to induce apoptosis in hepatocarcinoma cells²⁴ and a similar finding has been reported in renal cell carcinoma (RCC), where *in vitro* treatment of tumor cells with IL-4 and tumor necrosis factor- α is shown to increase RCC apoptosis.²⁵ However, this point remains controversial since several clinical trials of IL-4 therapy for the treatment of both hematologic and non-hematologic cancers have been disappointing, with little antitumor effects or even with an increase in the number of malignant cells.^{26–28} Conversely, it has recently been demonstrated that IL-4 may suppress cancer-directed immunosurveillance, facilitating pancreatic tumor growth and metastasis.¹⁵ Similarly, IL-4 has been more recently demonstrated to inhibit cell apoptosis, which could help in promoting tumor growth, increasing the expression of antiapoptotic genes cFLIP and Bcl-xL and protecting cells from CD95- and chemotherapy-induced apoptosis of cancer cells from prostate, breast and bladder cancers, following IL-4 *in vitro* treatment.²⁹

Moreover, we have previously shown that refractoriness of thyroid cancer cells to chemotherapy and death receptors is due to the concomitant production of IL-4 and IL-10.^{16,17} In the present study, we provide evidence that other epithelial tumors, including tumors of the colon, breast and lung, produce IL-4 in an autocrine manner. This cytokine is likely

to be associated with the high proliferation rate of tumor cells and with the inhibition of tumor cell apoptosis through upregulation of proteins that block the extrinsic as well as the intrinsic apoptotic pathway. Here, we demonstrate that inhibition of IL-4 reverses this resistance phenotype both *in vitro* and *in vivo*, suggesting that it may constitute a novel therapeutic strategy for the treatment of different types of epithelial neoplasia.

IL-4 is structurally related to IL-13, which competes for binding to its cell surface receptor exhibiting many similar biological effects. The molecular basis for this functional overlap has been attributed mainly to a shared use of the receptor complex for the IL-4 type II receptor, predominantly composed of the IL-4R α and IL-13R α 1.³⁰ Alternatively, IL-13 may bind with even greater affinity to IL-13R α 2, which fails to induce a signal, indicating that it acts as a decoy receptor.³¹

We thus investigated the expression of the IL-13 protein and its decoy IL-13R α 2. We found that IL-13 was undetectable in normal and cancer epithelial cells from colon and in breast tissue, while IL-13R α 2 was highly expressed in normal than in cancer colon cells and equally expressed in breast cells (data not shown). These data suggest that the protective effect observed in carcinoma cells is primarily due to the IL-4 autocrine production.

We and others have found that although the two apoptosis-inducing TRAIL receptors are expressed in epithelial cancer cells, including those from colon, breast and lung, their triggering often does not lead to a significant induction of cell death.^{32,33} In particular, here, we report that all the epithelial

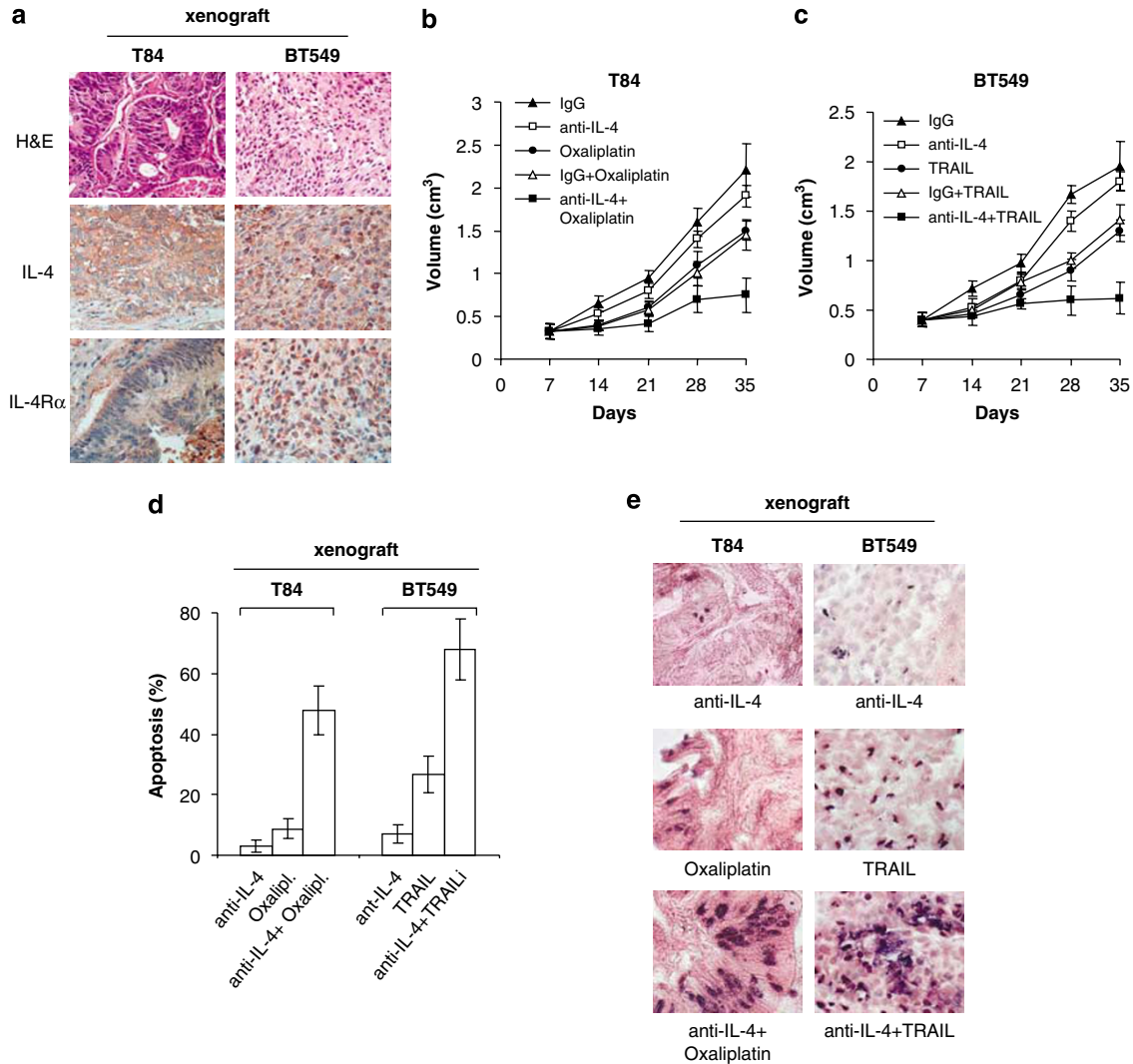


Figure 7 IL-4 neutralization sensitizes cancer cells to chemotherapy and to TRAIL-induced cell death *in vivo*. (a) H&E and immunohistochemical analysis of IL-4 on paraffin-embedded sections of tumors generated in nude mice by T84 and BT549 cancer cells (red staining) ($\times 40$ magnification). Nuclei were counterstained by hematoxylin (blue color). One representative of four independent experiments is shown. (b, c) *In vivo* growth of subcutaneous colon and breast carcinoma tumor xenografts after treatment with IgG or anti-IL-4 ($10 \mu\text{g}/\text{cm}^3$), alone or in combination with oxaliplatin (0.40 mg/kg) or TRAIL (30 mg/kg). Data are mean \pm S.D. of eight independent experiments. (d) Percentage of apoptotic events measured on sections of subcutaneous tumors (four per group) generated by T84 and BT549 and treated with anti-IL-4, oxaliplatin (oxalipl.), or TRAIL alone or with anti-IL-4 plus oxaliplatin or anti-IL-4 plus TRAIL and stained for TUNEL. Cells (5×10^5) per tumor were counted and the percentage of apoptotic event was calculated. Samples were analyzed six weeks after treatment had started. (e) A representative TUNEL staining on paraffin-embedded sections of tumor xenografts treated as in (c)

cancer cells examined are only partially sensitive to TRAIL and to chemotherapeutic drugs with a cell death rate ranging between 15 and 40%. High expression of antiapoptotic molecules is commonly found in human cancers and contributes to both neoplastic cell expansion and resistance to the therapeutic action of cytotoxic drugs and death receptor triggering.^{34,35} Several studies have shown that functional blockade of antiapoptotic factors can either restore the apoptotic process in tumor cells or sensitize them to chemotherapy- and death ligand-induced apoptosis.^{36,37} Accordingly, we showed that cancer cells express high levels of the antiapoptotic proteins PED/PEA-15, cFLIP, Bcl-xL and Bcl-2. This finding may explain why cancer cells despite expressing high levels of TRAIL-R1/R2 are refractory to

apoptosis induction, even in the presence of high concentrations of a highly active recombinant form of TRAIL, and why at the same time they are resistant to the effects of conventional chemotherapeutic drugs.

IL-4 neutralization reduced the growth rate of cancer cells *in vitro* and rendered them more susceptible to chemotherapy- and TRAIL-induced cell death, both *in vitro* and *in vivo*, indicating that this cytokine acts as a survival factor for epithelial cells.

Interestingly, *in vivo* treatment with anti-IL-4, chemotherapy or TRAIL alone was not sufficient to effectively prevent subcutaneous growth of colon and breast cancer cell line xenografts. However, when IL-4-neutralizing antibody was combined with chemotherapy or TRAIL, tumor growth was

considerably decreased and apoptotic events were significantly increased. Thus, inhibition of IL-4–IL-4R interaction reprograms cancer cells, exemplified by the concomitant downregulation of at least four antiapoptotic factors, rendering them sensitive to apoptosis induction by chemotherapy and TRAIL. Anti-IL-4 therapeutics have been tested in clinical trials of respiratory diseases, such as asthma and allergies, and no adverse effects were observed.³⁸ In addition, TRAIL has been used quite extensively in clinical trials even in combination with chemotherapeutics. The toxicity potential for the combinations of IL-4 antagonists with TRAIL and/or with chemotherapy is not yet known and needs to be carefully addressed in clinical trials.³⁹

One of the main physiological functions of IL-4 is to influence the immune system toward the production of T_H2 cells and suppression of the production of IFN- γ -producing T_H1 cells, the latter being important in efficient tumor immunity.⁴⁰ Such skewing of the immune system toward a T_H2 rather than a T_H1 response is reminiscent of a situation often encountered in cancer patients.⁴¹ Our data suggest that this diversion of the immune system may be another subversive consequence of the tumor cells' production of IL-4, which would again favor tumor cell survival by preventing the formation of a T_H1 environment that would be less favorable for them.

In conclusion, we provide evidence that IL-4 acts as a survival factor on epithelial cells by increasing the expression of antiapoptotic molecules rendering them resistant to the induction of apoptosis. We suggest that specific interference with signaling events triggered by IL-4R α crosslinking in combination with the currently used therapeutic approaches and/or with TRAIL receptor agonists may be used as a novel promising treatment option for patients affected by epithelial cancer.

Materials and Methods

Human tissues. Human colon, breast and lung tissue fragments were obtained, in accordance with the ethical standards of the institutional committee responsible for human experimentation of the University of Palermo, from 85 patients affected by colon adenocarcinoma (age range 55–70 years), 32 patients affected by ductal and lobular breast cancer (age range 40–55 years) and 9 patients affected by non-small cell lung cancer (NSCLC; age range 35–55 years). Histological diagnosis was based on the morphological microscopic features of carcinoma cells determining the histological type and grade. Grossly, all the colon tumors, except one that had a polypoid appearance, were ulcerative. On pathological staging, the infiltration was confined to the subserosa with no lymph node involvement in four cases (pT3, N0, M0 and Dukes B). Four cases showed infiltration of subserosa with lymph node involvement (pT3, N1, M0 and Dukes C), while in twelve cases nodal invasion and metastatic involvement of the liver were observed (pT4, N1, M1 and Dukes D). Seven of ten breast carcinomas analyzed were invasive ductal and three out of ten were invasive lobular. All the four NSCLCs analyzed were adenocarcinomas. Normal epithelial cells were obtained from the peri-tumoral tissues of the surgically removed specimens.

Human primary cell purification and apoptosis induction. Tissues from colon, breast and lung were digested for 2 h with collagenase (1.5 mg/ml) (Gibco BRL, Grand Island, NY, USA) and hyaluronidase (20 μ g/ml) (Sigma Chemical Co., St. Louis, MO, USA) as previously described.³⁵ Normal colon tissue specimens were extensively washed in DMEM and digested with collagenase P (0.4 U/ml) and dispase I (1.2 U/ml) for 4 min at 37°C. Crypt-like structures were obtained by mechanical dissociation. Epithelial cells were purified from the digested tissues by hematopoietic cell depletion with anti-CD45-coupled beads (*Dynal*; Wirral Merseyside, UK) followed by 12 h of flask adherence, which allowed removal

of other cells. Fibroblasts were depleted exposing cell cultures to trypsin + EDTA for 1 min.

Once digested, cells were maintained on plastic in DMEM medium (Euroclone Ltd., West York, UK) at 37°C in a humidified atmosphere of 5% CO₂. After an additional 12 h, cells were allowed to grow in a monolayer. Cells were detached with trypsin in 5 mM EDTA for functional protein expression and gene transcript level analyses. Colon cells were cultured on plastic flask pre-coated with collagen at 10 μ g/cm² (Calbiochem GmbH, Darmstadt, Germany). Oxaliplatin (0–200 μ M), doxorubicin (0–20 μ M) (Sigma Chemical Co.) and etoposide (0–20 μ M) (Biomol, Plymouth Meeting, PA, USA) were used to determine time-course (6–48 h) and dose–response curves in primary human epithelial cancer cells while iz-TRAIL (0–20 μ g/ml)⁴² treatment for 12 h was used to determine a dose–response curve in the same cells. T98G TRAIL-sensitive cell line was used as a positive control of TRAIL treatment. Primary cells from normal tissues and from cancerous lesions were cultured up to 15 and 12 days, respectively, in the presence or absence of human recombinant IL-4 (20 ng/ml) (Euroclone, Paignton, UK) or 10 μ g/ml neutralizing Ab against human IL-4 (3007.11 mouse IgG₁; R&D Systems Europe Ltd). The pretreatment of primary cancer cells with anti-IL-4 Ab was also used to test the sensitization to the chemotherapeutic drugs of primary cancer cells.

The T84 human colon carcinoma cell line and the BT549 human ductal breast carcinoma cell line were cultured in DMEM/F12 with 10% FCS and RPMI 1640 with 5% FCS, respectively, in the presence or absence of anti-IL-4 Ab in combination with oxaliplatin (100 μ M) and TRAIL (200 ng/ml), respectively.

Survival and death assays. To evaluate apoptotic events, annexin V/propidium iodide staining was performed and analyzed by flow cytometry. Alternatively, human purified cancer cells were plated in 96-well plates in triplicate at 15 000 cells per well and cultured for the indicated periods of time. The number of viable cells was detected by CellTiter Aqueous Assay Kit (Promega Corporation, WI, USA) following the manufacturer's instructions. HuT78 cells plated at 2×10^5 /ml and treated with an agonistic anti-CD95 Ab (CH-11; 200 ng/ml) were used as a positive control for cell death measurement.

Immunohistochemical analysis. Immunohistochemistry was performed on 6- μ m-thick paraffin-embedded sections of human colon, breast and lung normal or tumor samples or of subcutaneous tumors generated in nude mice by injection of T84 or BT549 tumor cells. Dewaxed sections were treated for 10 min in a microwave oven in 0.1 M citrate buffer. Then, sections were incubated for 10 min with Tris-buffered saline (TBS) containing 10% AB human serum to block unpecific binding. After elimination of excess serum, sections were exposed overnight at 4°C to specific Abs against TRAIL-R1 (TR1.02 mouse IgG₁), TRAIL-R2 (TR2.21 mouse IgG₁), IL-4 (B-S4 mouse IgG₁; Caltag Laboratories, Burlingame, CA, USA), IL-4R α (C-20 rabbit IgG; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or isotype-matched controls at appropriate dilutions. Following exposure to primary Abs, sections were treated with biotinylated anti-rabbit or anti-mouse immunoglobulins, washed in TBS and then incubated with streptavidin peroxidase (LSAB 2 Kit; Dako Corporation, Carpinteria, CA, USA). Staining was detected using 3-amino-9-ethylcarbazole as a colorimetric substrate. Counterstaining of cells was performed using aqueous hematoxylin.

Protein isolation and western blotting analysis. 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 and phosphatase inhibitors as previously described.¹³ Immunoblotting of Abs specific for PED/PEA-15 (rabbit IgG), cFLIP (NF6 mouse IgG₁; Alexis Biochemicals, Switzerland), Bcl-xL (H-5, mouse IgG₁; Santa Cruz Biotechnology Inc.), Bcl-2 (124, mouse IgG₁; Upstate Biotechnology Inc.) and actin (Ab-1, mouse IgM; Calbiochem GmbH) was detected by HRP-conjugated anti-mouse or anti-rabbit Abs (Amersham Biosciences UK Limited, England) and visualized by a chemiluminescence detection system (SuperSignal West Dura Extended Duration Substrate; Pierce, IL, USA).

Real-time PCR analysis. Total RNA was prepared from cultured cells using the RNeasy Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's instruction. Reverse transcription of total RNA was performed using the High-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Quantitative TaqMan PCR analysis was carried out with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) in a reaction volume of 25 μ l containing 1 \times TaqMan Universal Master Mix (Applied Biosystems, Branchburg, NJ, USA) and 1 \times probes and primer sets Hs00174122_m1 (IL-4) (Taqman Gene Expression

Assays; Applied Biosystems, Foster City) or $1 \times$ Hu GAPDH (Pre-Developed TaqMan Assay Reagents; Applied Biosystems, Warrington, UK). Reactions were performed using the following thermal cycler parameters: incubation at 50°C for 2 min and denaturation at 95°C for 10 min, then 45 cycles of the amplification step (denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min).

All amplification reactions were performed in triplicate and the relative quantification of IL-4 gene expression was expressed after normalization with the endogenous control, GAPDH. Data processing and statistical analysis were performed using the ABI PRISM SDS, software version 2.1 (Applied Biosystems, Foster City).

ELISA. IL-4 levels in primary cancer cells and T84 and BT549 cell lines were measured by quantitative sandwich ELISA, using a commercially available ELISA kit (Human Interleukin 4 ELISA; Euroclone). The minimum detectable dose of IL-4 was 0.5 pg/ml. Streptavidin peroxidase was added after biotinylated anti-IL-4. The chromogen used was tetramethyl benzidine and the reaction was stopped by using H₂SO₄. Optical density readings were performed at 450 nm and corrected at 540 nm using GDV programmable MPT reader DV990BV6.

Flow cytometry analysis. For FACS analysis of IL-4 and IL-4R α expression on T84 and BT549 cells, R-PE-conjugated anti-human IL-4 rat monoclonal Ab (Caltag Laboratories) and R-PE-conjugated anti-human IL-4R α Ab (hIL4R-M57 mouse IgG1; BD Pharmingen, CA, USA) were used. Jurkat cells, exposed for 4 h to PMA (10 ng/ml) and ionomycin (1 μ M), were used as a positive control.

Animals and tumor system. Nude mice were purchased from Charles River Laboratories (Milan, Italy), housed 3–4 per cage and acclimatized to the animal housing facility for a minimum period of 1 week before beginning the experiments. All the experiments were carried out according to current regulations, observing the interdisciplinary principles and guidelines for the use of animals in research, marketing and education. Mice received subcutaneous injection of 2×10^6 T84 human colon cancer cells or 2×10^6 BT549 human ductal breast cancer cells in 200 μ l of PBS and after 7 days, when well-established tumors of about 0.3 cm³ were detected, 10 mice per group were treated i.p. with oxaliplatin alone (0.40 mg/kg on day 1 every week for 4 weeks) or with TRAIL alone (30 mg/kg once per day for five consecutive days followed by a once weekly dose until they were killed) or in combination with anti-IL-4 (R&D Systems Europe Ltd; 10 μ g/cm³ on day 1 and day 4 for 3 weeks) inoculated into the tumor. Controls were assessed using IgG isotype-matched control or PBS. Tumors were measured weekly with a calliper and volumes were calculated on the basis of the formula: $(\pi/6) \times$ larger diameter \times (smaller diameter)².

TUNEL staining. TUNEL reaction was performed on paraffin-embedded sections of subcutaneous tumors generated by T84 or BT549 cell lines (10 μ m). Sections were post-fixed with paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Apoptosis was determined by *In Situ* Cell Death Detection AP Kit (Boehringer Mannheim, Indianapolis, IN, USA). DNA strand breaks were detected by 5-bromo-4-chloro-3-indolyl-phosphate (Dako Corporation) substrate. Positive control was performed by pretreating specimens with DNase I (1 μ g/ml) to introduce nonspecific strand breaks, while the negative control was subjected to the same staining for TUNEL without TdT.

Statistical analysis. Percentage of apoptotic events was calculated from the values of the MTS assay and annexin V/propidium iodide staining. Data were expressed as mean \pm S.D. S.D. was calculated by the square root of the arithmetic mean of the squares of the deviations from the arithmetic mean. 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). FLIP_L analysis was carried out by summing up the densities of the bands at 55 and 43 kDa (for FLIPs, when detected) and then dividing by the density of the β -actin band. Results were expressed as protein/ β -actin OD ratio (OD).

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