

IL-4 Protects Tumor Cells from Anti-CD95 and Chemotherapeutic Agents via Up-Regulation of Antiapoptotic Proteins¹

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We recently proposed that Th1 and Th2 cytokines exert opposite effects on the pathogenesis and clinical outcome of organ-specific autoimmunity by altering the expression of genes involved in target cell survival. Because a Th2 response against tumors is associated with poor prognosis, we investigated the ability of IL-4 to protect tumor cells from death receptor- and chemotherapy-induced apoptosis. We found that IL-4 treatment significantly reduced CD95 (Fas/APO-1)- and chemotherapeutic drug-induced apoptosis in prostate, breast, and bladder tumor cell lines. Analysis of antiapoptotic protein expression revealed that IL-4 stimulation resulted in up-regulation of cellular (c) FLIP/FLAME-1 and Bcl-x_L. Exogenous expression of cFLIP/FLAME-1 inhibited apoptosis induced by CD95 and to a lesser extent by chemotherapy, while tumor cells transduced with Bcl-x_L were substantially protected both from CD95 and chemotherapeutic drug stimulation. Moreover, consistent IL-4 production and high expression of both cFLIP/FLAME-1 and Bcl-x_L were observed in primary prostate, breast, and bladder cancer in vivo. Finally, primary breast cancer cells acquired sensitivity to apoptosis in vitro only in the absence of IL-4. Thus, IL-4 protects tumor cells from CD95- and chemotherapy-induced apoptosis through the up-regulation of antiapoptotic proteins such as cFLIP/FLAME-1 and Bcl-x_L. These findings may provide useful information for the development of therapeutic strategies aimed at restoring the functionality of apoptotic pathways in tumor cells. *The Journal of Immunology*, 2004, 172: 5467–5477.

The ability of some neoplastic cells to escape the immune response leads to tumor development. Traditional anti-neoplastic therapy is based on the use of chemotherapeutic compounds, which exert a cytotoxic effect on proliferating cells and promote the destruction of sensitive tumors (1). Apoptosis is the predominant mechanism by which cancer cells die in response to immune attack or to cytotoxic drugs (2, 3). Therefore, the development of effective antiapoptotic mechanisms can result in malignant cell resistance to immune reaction and therapy (4).

Apoptosis signaling initiated by death receptors or anticancer drugs proceeds through the generation of caspase- and mitochondria-mediated events that result in ultimate cell demise (3). Triggering of CD95 (Fas/APO-1) or other death receptors leads to the recruitment of the adaptor molecule Fas-associated death domain protein, which binds directly to the receptor death domain and recruits procaspase-8 to form a death-inducing signaling complex

(DISC)³ (5). Proteolytic activation of procaspase-8 is inhibited by cellular (c) FLIP/Fas-associated death domain-like antiapoptotic molecule (FLAME) 1, which blocks death receptor-mediated events at the DISC level, thus preventing the activation of the downstream caspase cascade (6). The cFLIP/FLAME-1 protein, also called caspase homolog, Casper, caspase-like apoptosis regulatory protein, I-FLICE, MACH-related inducer of toxicity, and Usurpin, is structurally similar to caspase-8 as it contains two death effector domains and a caspase-like domain, but lacks the key residues for proteolysis, most notably the cysteine within the active site (7). Hence, high expression of cFLIP/FLAME-1 prevents death receptor-induced apoptosis. A number of death stimuli promote the release from mitochondria of cytochrome *c*, apoptosis-inducing factor, Apaf-1, Smac/direct IAP binding protein with low pI, and possibly other apoptotic factors (3). Cytochrome *c* associates with Apaf-1 and caspase-9 to form the so-called apoptosome, which activates downstream caspases responsible for the modification of protein substrates within the nucleus and the cytoplasm, thus leading to the execution phase of apoptosis (8). Bcl-2 family proteins modulate apoptosis by controlling permeabilization of the outer mitochondrial membrane (9). Antiapoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-x_L, stabilize the mitochondrial barrier function and inhibit cytochrome *c* release, whereas proapoptotic Bcl-2 proteins antagonize this process by competitive heterodimerization with antiapoptotic members of the family (10, 11). A dysregulated expression of antiapoptotic components of the cell death machinery has been reported to result in resistance of

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³ Abbreviations used in this paper: DISC, death-inducing signaling complex; FLAME, Fas-associated death domain-like antiapoptotic molecule; GFP, green fluorescent protein; LNCaP, lymph node metastasis of prostate adenocarcinoma; MAP, mitogen-activated protein; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; TIL, tumor-infiltrating lymphocyte; cFLIP, cellular FLIP.

cancer cells to apoptosis, thus favoring tumor progression (4). Increased expression of Bcl-2 family members or cFLIP/FLAME-1 has been detected in tumor cells and is believed to correlate with the resistance of neoplastic cells to chemotherapeutic drugs (4, 12–14).

The antitumor immune response is predominantly sustained by tumor-infiltrating T lymphocytes (TILs), consisting of CD4⁺ and CD8⁺ T cells (15). CD4⁺ Th lymphocytes have been divided into Th1 and Th2 subpopulations, based on their functional properties and on the profile of secreted cytokines. Th1 cells are responsible for the production of IFN- γ , IL-2, and TNF- β , while Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 (16). The balance between Th1 and Th2 subpopulations of TILs has received considerable attention, as it has been reported that Th2 cytokine production is high in a large percentage of advanced tumors and inversely correlated with good prognosis (17). Specifically, high levels of IL-4 production have been detected among the TILs of several advanced solid cancers (17). The role of Th cytokines is not confined to the regulation of the immune response. We recently proposed that Th1 and Th2 cytokines exert opposite effects on cells targeted by the immune response through the direct modulation of key apoptosis-related genes that promote or inhibit target cell survival (18). IL-4 has been reported to induce apoptosis resistance in chronic lymphocytic leukemia B cells, while being able to enhance antiapoptotic protein expression in normal and transformed lymphocytes and in thyroid cells (19, 20). Although a decreased Th1/Th2 ratio among TILs may impair their cytotoxic activity against tumor cells, we speculated that Th2 cytokines do not simply act through the modulation of the immune response, but they may promote cancer cell survival through the up-regulation of antiapoptotic genes.

In this view, the antiapoptotic activity of Th2 cytokines may have detrimental effects in cancer. To examine a possible correlation between IL-4-mediated antiapoptotic effects and the malignancy of Th2-infiltrated tumors, we investigated the ability of IL-4 to modulate apoptosis and to influence levels of apoptosis-related proteins in cells derived from solid tumors. Prostate, bladder, and breast carcinoma cells were selected for this study because patients with these cancers have high levels of Th2 cytokines in the peripheral blood and in the tumor microenvironment (21–24). In this study, we report that IL-4 strongly inhibits chemotherapy- and CD95-induced apoptosis through the up-regulation of the antiapoptotic proteins Bcl-x_L and cFLIP/FLAME-1, suggesting that IL-4 production may contribute to apoptosis resistance of human solid tumors during Th2 responses.

Materials and Methods

Cell culture and reagents

Human lymph node metastasis of prostate adenocarcinoma (LNCaP) prostate cancer cell line and human MDA-MB-231 breast cancer cell line were obtained from the American Type Culture Collection (Manassas, VA). Human RT112 bladder carcinoma cell line was kindly provided by M. Cipitelli (Regina Elena Cancer Institute, Rome, Italy). Cells were grown in RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% heat-inactivated FBS supplemented with 2 mM L-glutamine and 100 U/ml penicillin-streptomycin. Cells were kept in a 5% CO₂ atmosphere and routinely passaged when 80–85% confluent.

Human rIL-2 and rIL-4 were purchased from PeproTech (Rocky Hill, NJ). Antineoplastic agents were purchased from Sigma-Aldrich (St. Louis, MO) and resuspended in DMSO (cisplatin, camptothecin, and etoposide) or in H₂O (daunorubicin and vincristine). Camptothecin (50 ng/ml), cisplatin (300 ng/ml), daunorubicin (5 μ M), etoposide (7 μ M), Taxol (5 μ M), and vincristine (1 μ M) were used in vitro at doses compatible with the levels reached in vivo during cancer treatment. Anti-CD95 agonistic Ab (clone CH11) was purchased from Upstate Biotechnology (Lake Placid, NY) and used at 30 ng/ml. mAb anti-Bcl-x_L (H5, mouse) was from Santa Cruz Biotechnology (Santa Cruz, CA); Ab anti-cFLIP/FLAME-1 (NF6,

mouse) was kindly provided by P. Krammer (German Cancer Research Center, Heidelberg, Germany). Ab anti- β -actin (goat) was purchased from Oncogene (Boston, MA). Normal and cancer tissues from breast, prostate, and bladder specimens were digested for 3 h with collagenase (1.8 mg/ml) (Life Technologies) and hyaluronidase (25 μ g/ml) (Sigma-Aldrich) in DMEM. After 12 h of culture, normal and cancer cells were detached with trypsin plus EDTA following exposure to cytokines or chemotherapeutic agents for functional and protein analyses. Cells were cultured in standard DMEM with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT) in the presence or absence of human recombinant cytokines.

Cell viability assay

Cell viability was determined using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI), according to the manufacturer's instructions. The assay is based on reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) to a colored formazan product that is measured spectrophotometrically. Cells were seeded in 96-well tissue culture plates and incubated at 37°C in a 5% CO₂ incubator overnight. The next day, IL-2 or IL-4 was added at a concentration of 20 ng/ml. After 2 days, cells were treated for 10 h with chemotherapeutic drugs or anti-CD95, and then 20 μ l of MTS was added to each well. After 3 h of incubation at 37°C with the MTS reagent, the plates were read on a Multilabel Counter (Victor2, Wallac; PerkinElmer, Norwalk, CT), and dye absorbance was measured at 490 nm.

Western blotting

Cell pellets were washed twice with cold PBS and lysed on ice for 30 min with 1% Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.2, 200 mM NaCl, 1% Nonidet P-40) in the presence of 1 mM PMSF and 2 μ g/ml each of aprotinin, leupeptin, and pepstatin. Cell debris was removed by centrifugation at 13,000 rpm for 10 min at 4°C. Lysate concentration was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Aliquots of cell extracts containing 30 μ g of total protein were resolved on 10% or 12% SDS-PAGE and transferred to a Hybond-C extra nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ).

Filters were blocked for 1 h at room temperature in 5% nonfat dry milk dissolved in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.2% Tween 20) and then incubated in 1% BSA/TBST containing a dilution of primary Ab (1/200 anti-Bcl-x_L, 1/10 anti-cFLIP, and 1/10,000 anti- β -actin) for 3 h (Bcl-x_L and β -actin) or overnight (cFLIP). After washing in TBST buffer, filters were incubated for 45 min in 5% nonfat dry milk dissolved in TBST containing a 1/4000 dilution of corresponding peroxidase-conjugated secondary Ab (Amersham Pharmacia Biotech). Proteins were visualized with the ECL technique (Super Signal West Pico; Pierce, Rockford, IL). Image processing and analysis were performed with the NIH IMAGE software version 1.62 (by W. Rasband, National Institutes of Health, Research Services Branch, Alcohol, Drug Abuse, and Mental Health Administration).

Immunostaining procedure

Immunohistochemical analyses were performed on 7- μ m-thick paraffin-embedded sections. Deparaffinized sections were treated for 10 min in a microwave oven in 0.1 M citrate buffer. Following elimination of excess serum, sections were exposed for 1 h to specific Abs against Bcl-x_L (H-5, mouse IgG1; Santa Cruz Biotechnology), CD45RO (UCHL1, mouse IgG2a; DAKO, Carpinteria, CA), IL-4 (B-S4 mouse IgG1; Caltag Laboratories, Burlingame, CA), cFLIP (rabbit polyclonal IgG; Upstate Biotechnology), IL-4R (C-20, rabbit polyclonal IgG; Santa Cruz Biotechnology), or isotype-matched controls at appropriate dilutions. After two washes in TBS, sections were treated with biotinylated anti-rabbit or anti-mouse Igs, washed in TBS, and incubated with streptavidin peroxidase (DAKO LSAB 2 kit). Staining was detected using 3-amino-9-ethylcarbazole as a colorimetric substrate. Counterstaining of cells and tissue sections was performed using aqueous hematoxylin.

Production of retroviral particles and infection of cell lines

cFLIP and Bcl-x_L cDNAs were cloned into the PINCO retroviral vector carrying the green fluorescent protein (GFP) as a reporter gene (25). The amphotropic packaging cell line Phoenix was transfected with the PINCO-1/Bcl-x_L, PINCO-1/cFLIP plasmids, or with the empty vector by standard calcium-phosphate-chloroquine method. Culture supernatants containing viral particles were collected after 48 h, filtered (0.45 μ m), and added to 3 \times 10⁵ cells plated on six-well plates. For one cycle of infection, cells were centrifuged at 1800 rpm for 45 min at 32°C and kept in the incubator for 75 min. Cells were subjected to two infection cycles each day for 2

consecutive days and then placed in standard medium. GFP-positive cells were analyzed by flow cytometry 24 h after the last infection cycle (FACSscan; BD Biosciences, San Jose, CA).

Statistical analysis

The percentage of apoptotic cells was derived from the percentage of viable cells that was directly calculated from the values of the MTS assay.

The percentage of protection from apoptosis was determined as: % protection = 100% - X. X was calculated with the formula: (C1-C2) : 100 = (S1-S2) : X, where: C1 is the viability of untreated control cells, C2 is the viability of control cells treated with the apoptotic stimulus, S1 and S2 are the viability of cells preincubated with cytokines or transduced with anti-apoptotic genes, untreated (S1) or treated (S2) with the apoptotic stimuli.

Paired *t* test was used to analyze the statistical significance of the experimental results. Values of *p* < 0.05 were considered significant. Data are presented as mean values ± 1 SD of the mean.

Results

IL-4 inhibits chemotherapy- and CD95-induced apoptosis in bladder cancer cells

A decreased Th1/Th2 ratio has been observed in a wide variety of human cancers, in which it has been proposed to correlate with the stage and grade of malignancy (17). IL-4 released from tumor-associated Th2 lymphocytes has been shown to promote the growth of pulmonary metastatic cancer in mice (26), suggesting a specific role of this cytokine in influencing tumor cell survival. Therefore, we investigated the ability of IL-4 to modulate apoptosis sensitivity of cells derived from different human solid tumors.

Anticancer treatment using cytotoxic drugs mediates cell death by activating the intracellular apoptotic program (3). To determine

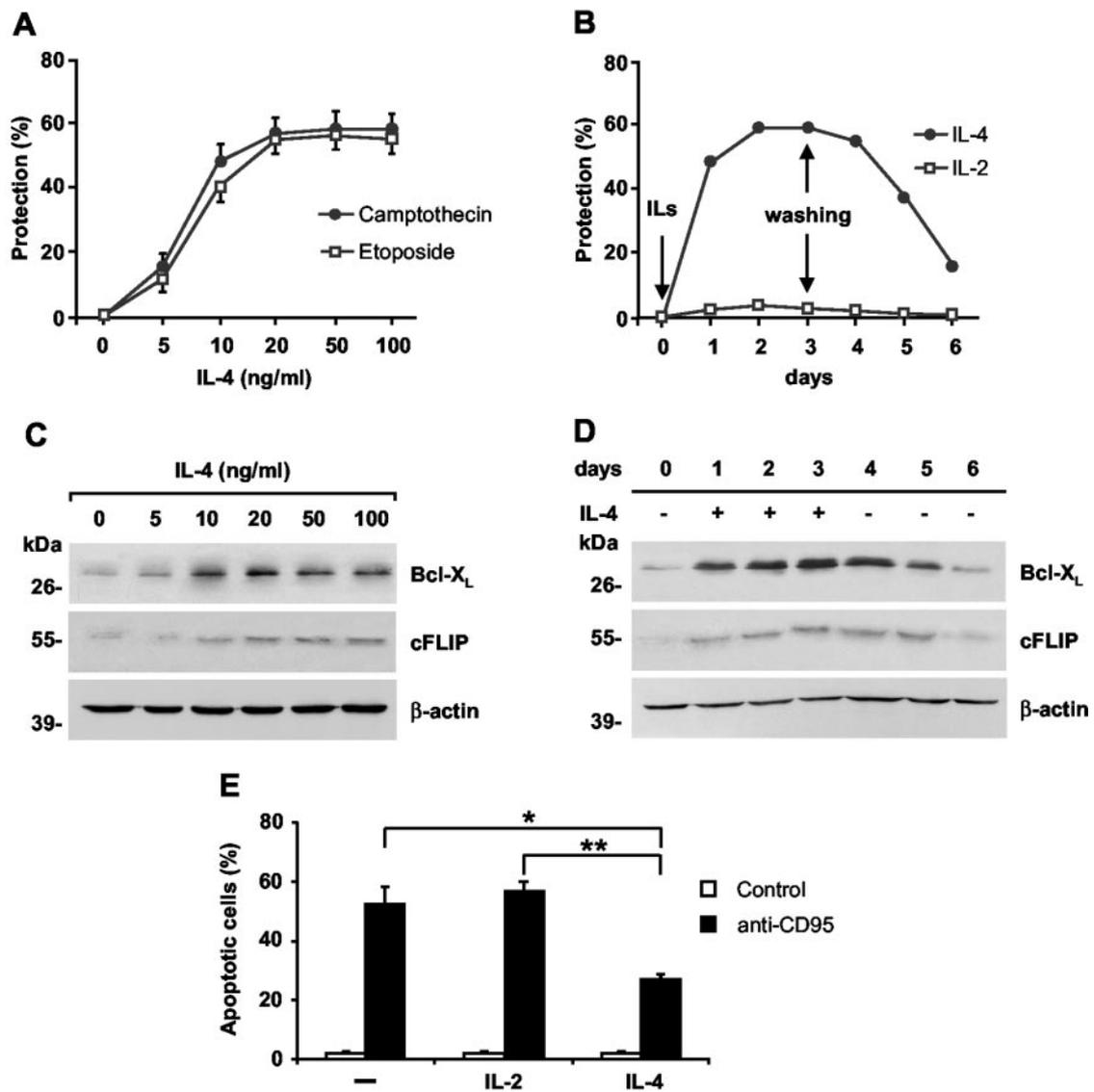


FIGURE 1. IL-4 increases bladder cancer cell survival and up-regulates Bcl-x_L and cFLIP/FLAME-1. *A*, Protective effects of 2-day treatment with increasing doses of IL-4 on RT112 bladder carcinoma cells exposed for 24 h to camptothecin or etoposide. The results shown are the mean ± SD of four independent experiments. *B*, Protective effects of 20 ng/ml IL-2 or IL-4 on RT112 bladder carcinoma cells untreated (0) or treated for 1, 2, or 3 days and exposed for 24 h to etoposide. IL-2 and IL-4 (ILs) were added on day 0 and removed by washing on day 3. The percentage of protection was calculated vs cells not treated with cytokines. *C*, Immunoblot analysis of Bcl-x_L and cFLIP/FLAME-1 expression levels in RT112 treated as in *A*. *D*, Immunoblot analysis of Bcl-x_L and cFLIP/FLAME-1 expression levels in RT112 treated as in *B*. One representative experiment of three performed is shown. *E*, Percentage of apoptotic RT112 cells untreated or pretreated for 2 days with 20 ng/ml IL-4 or IL-2 and incubated with 30 ng/ml anti-CD95 agonistic Ab. The results shown are the mean ± SD of four independent experiments. *, Indicates *p* < 0.05; **, indicates *p* < 0.01.

whether IL-4 was able to inhibit apoptosis induced by chemotherapeutic drugs, bladder tumor cells (RT112) were pretreated for 2 days with different concentrations of IL-4 and subsequently exposed to camptothecin or etoposide. Although a considerable number (~60%) of tumor cells underwent apoptosis in response to chemotherapeutic agents, cells preincubated with IL-4 were significantly protected from drug-induced death (Fig. 1A), suggesting that IL-4 interferes with the apoptotic program activated by anticancer agents in tumor cells. The protective effect of IL-4 was already consistent after 24 h of pretreatment and remained stable from the second day of treatment unless IL-4 was removed from the culture medium (Fig. 1B, and data not shown).

The observation that tumor cells treated with IL-4 display a reduced sensitivity to chemotherapeutic drugs led us to investigate whether this cytokine would influence the expression of apoptosis regulatory genes. Western blot analysis of apoptosis-related proteins was performed on cancer cell lines treated for 48 h with IL-4 as compared with untreated or IL-2-treated controls. Among the proteins examined, we found that levels of Bcl-x_L and cFLIP/FLAME-1 were increased in tumor cells treated with IL-4, while levels of caspases and other pro- and antiapoptotic Bcl-2 family members remained unchanged (Fig. 1, C and D, and data not shown). The increased expression of cFLIP/FLAME-1 prompted us to investigate whether IL-4 could protect tumor cells from death receptor stimulation. CD95 ligand (CD95L) is one of the major effector molecules of CTL and NK cells (27). The CD95 pathway has been demonstrated to be involved in tumor clearance in vivo (13). CD95 mutation or down-modulation has been found in sev-

eral tumors (28, 29), and the development of resistance to CD95-induced cell death has been suggested to contribute to immune evasion of malignant cells (30). As shown in Fig. 1E, IL-4 treatment was able to significantly inhibit CD95-induced apoptosis in bladder cancer cells, suggesting that IL-4 can negatively affect the immune response against tumors.

IL-4 inhibits chemotherapy- and CD95-induced apoptosis in prostate and breast cancer cells

We hypothesized that the ability of IL-4 to protect bladder cancer cells from chemotherapy- and anti-CD95-induced apoptosis also could be displayed by other tumors characterized by the presence of IL-4, such as prostate and breast cancer. Therefore, tumor cell lines derived from prostate (LNCaP) and breast (MDA-MB-231) carcinomas were pretreated for 2 days with IL-4 and subsequently exposed to those antineoplastic agents that showed the highest cytotoxic activity toward each cell line. Although a considerable percentage of prostate and breast tumor cells cultured in medium alone or pretreated with IL-2 underwent apoptosis in response to chemotherapeutic agents, cells preincubated with IL-4 were significantly protected from drug-induced death (Fig. 2, A and B). Similarly, IL-4 greatly reduced anti-CD95-induced apoptosis (Fig. 2C), suggesting that the presence of IL-4 in the tumor infiltrate may protect cancer cells from cytotoxic therapy and immune response. Conversely, pretreatment of cells with IL-2 did not exert any protective effect, demonstrating the specificity of IL-4-mediated signals in the inhibition of apoptosis initiated by CD95 in

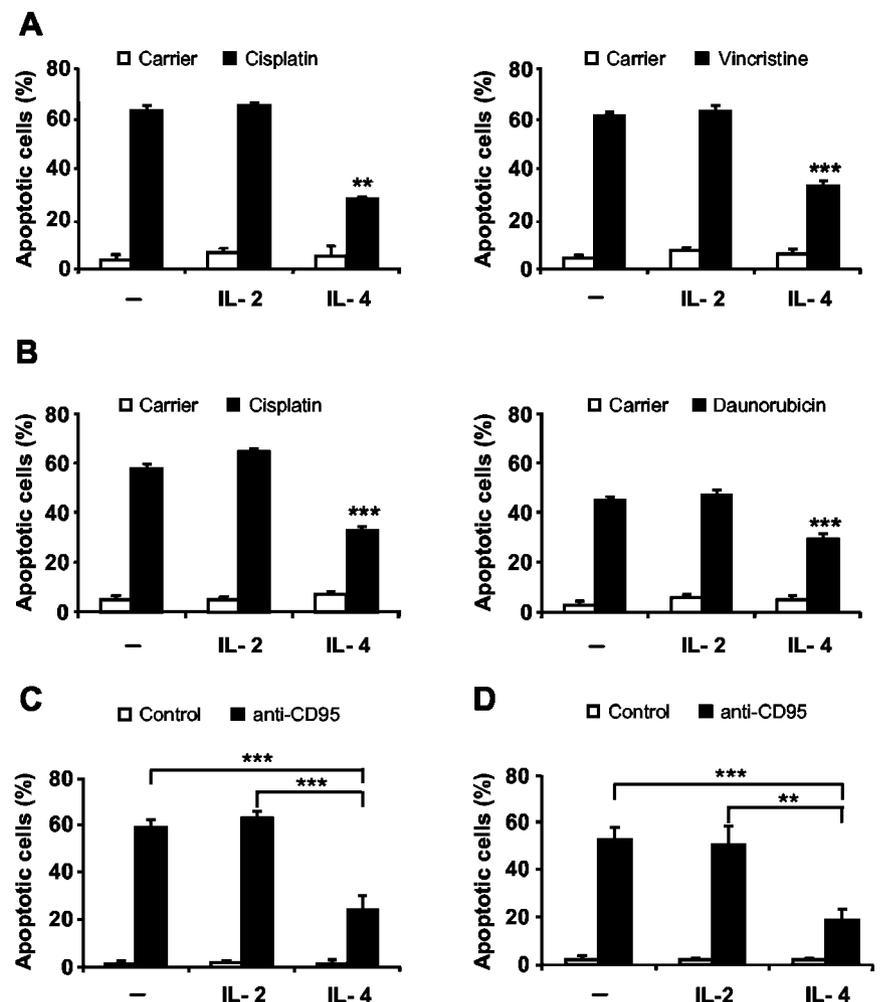


FIGURE 2. IL-4 prevents apoptosis induced by chemotherapeutic drugs in prostate and breast cancer cells. Percentage of apoptosis in LNCaP prostate cancer cells (A and C) and MDA-MB-231 breast cancer cells (B and D) pretreated for 2 days with 20 ng/ml IL-4 or IL-2 and exposed for 24 h to chemotherapeutic drugs or anti-CD95. Control cells (-) were stimulated with chemotherapeutic drugs (A and B) or anti-CD95 (C and D) in the absence of cytokine pretreatment. The results shown are the mean \pm SD of four independent experiments. **, Indicates $p < 0.01$; ***, indicates $p < 0.001$. In A and B, the same statistical significance was observed vs control and IL-2.

cancer cells. Notably, cells pretreated with IL-4 not only displayed an increased survival to cytotoxic drugs and CD95 stimulation, but regained full proliferative activity when the cytotoxic stimulus was removed (data not shown). Therefore, it appears that IL-4 is able to exert a significant effect on survival and growth of tumor cells, including a faster expansion of cells that survive chemotherapy treatment.

IL-4 up-regulates the expression of antiapoptotic proteins in prostate and breast cancer cells

The observation that prostate and breast tumor cells treated with IL-4 display a reduced sensitivity to death induced by CD95 and chemotherapeutic drugs led us to investigate whether this cytokine would influence the expression of apoptosis regulatory genes. As for bladder tumor cells, we found that levels of Bcl-x_L and cFLIP/FLAME-1 were considerably up-regulated following IL-4 treatment in LNCaP prostate cancer cells (Fig. 3A), thus providing a possible explanation for IL-4-mediated protection from apoptotic events initiated by chemotherapy and CD95 receptor. Differently, only a modest increase in cFLIP/FLAME-1 was observed in MDA-MB-231 cells, in which Bcl-x_L most likely represents the primary effector of IL-4-mediated protection from apoptosis (Fig. 3B). Therefore, IL-4-mediated increased survival in bladder, prostate, and breast cancer cells is associated with up-regulation of antiapoptotic proteins.

Exogenous expression of Bcl-x_L or cFLIP/FLAME-1 protects tumor cells from drug-induced apoptosis

Antiapoptotic Bcl-2 family members regulate the release of cytochrome *c* from mitochondria and have been implicated in apoptosis modulation of tumor cells exposed to chemotherapeutic drugs (10, 31–33). Increased expression of cFLIP/FLAME-1 has been reported to inhibit CD95-dependent apoptosis of malignant cells, thus resulting in tumor escape from T cell immunity *in vivo* (13). To determine whether increased expression of these two antiapoptotic effectors was able to protect tumor cells from apoptosis induced by chemotherapeutic drugs, we transduced RT112, MDA-MB-231, and LNCaP cells with a retroviral vector containing the cDNA for Bcl-x_L or cFLIP/FLAME-1 and the GFP as a reporter gene. Retroviral infection yielded 100% GFP-positive cell populations, which were then analyzed for expression of the transduced genes. By titrating viral supernatant and modulating the number of infection cycles, we were able to obtain levels of Bcl-x_L and cFLIP/FLAME-1 in transduced cells comparable to those of IL-4-treated cells, as shown by immunoblot analysis and densitometry quantification (Fig. 3, C–E). Cells were then exposed to chemotherapeutic drugs, and protection from apoptosis exerted by exogenous Bcl-x_L or cFLIP/FLAME-1 was evaluated in relation to non-transduced cells pretreated with IL-4 or with the control cytokine IL-2. Whereas control cells were efficiently killed by cytotoxic drugs, exogenous expression of Bcl-x_L protected tumor cells from drug-induced apoptosis at levels similar to those produced by IL-4 (Fig. 4). A partial inhibition of drug-induced death was also observed in cells overexpressing cFLIP/FLAME-1 (Fig. 4), possibly reflecting the ability of cFLIP/FLAME-1 to activate antiapoptotic signals through NF- κ B (34) or alternatively indicating the involvement of death receptor-mediated events in chemotherapy-induced apoptosis (35).

Exogenous expression of Bcl-x_L or cFLIP/FLAME-1 protects tumor cells from CD95-induced apoptosis

Bcl-x_L and cFLIP/FLAME-1 have both been demonstrated to interfere with apoptotic signals initiated at the CD95 receptor, although with different mechanisms. cFLIP/FLAME-1 inactivates

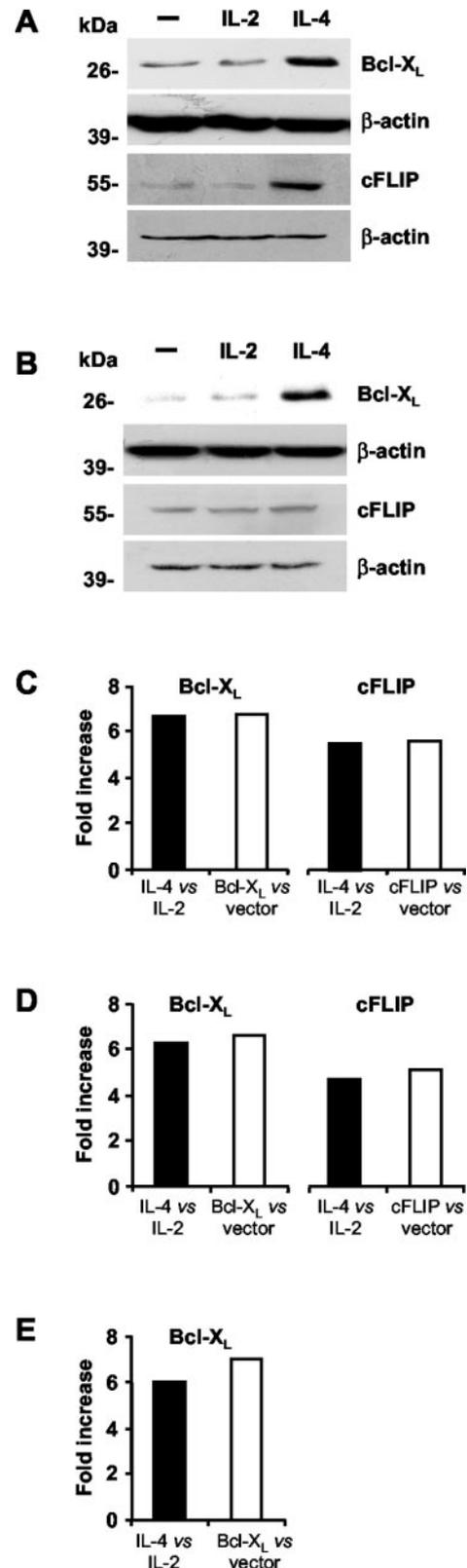


FIGURE 3. IL-4 up-regulates Bcl-x_L and cFLIP/FLAME-1 expression levels. Immunoblot analysis of Bcl-x_L and cFLIP/FLAME-1 (cFLIP) levels in LNCaP (A) and MDA-MB-231 (B). One representative experiment of four performed is shown. Comparison of Bcl-x_L and cFLIP/FLAME-1 expression in RT112 (C), LNCaP (D), and MDA-MB-231 (E) treated with IL-4 or transduced with the exogenous gene. Data are expressed as ratio between the protein level obtained in cells treated with IL-4 or transduced with the relevant gene and in cells treated with IL-2 or transduced with the empty vector, respectively.

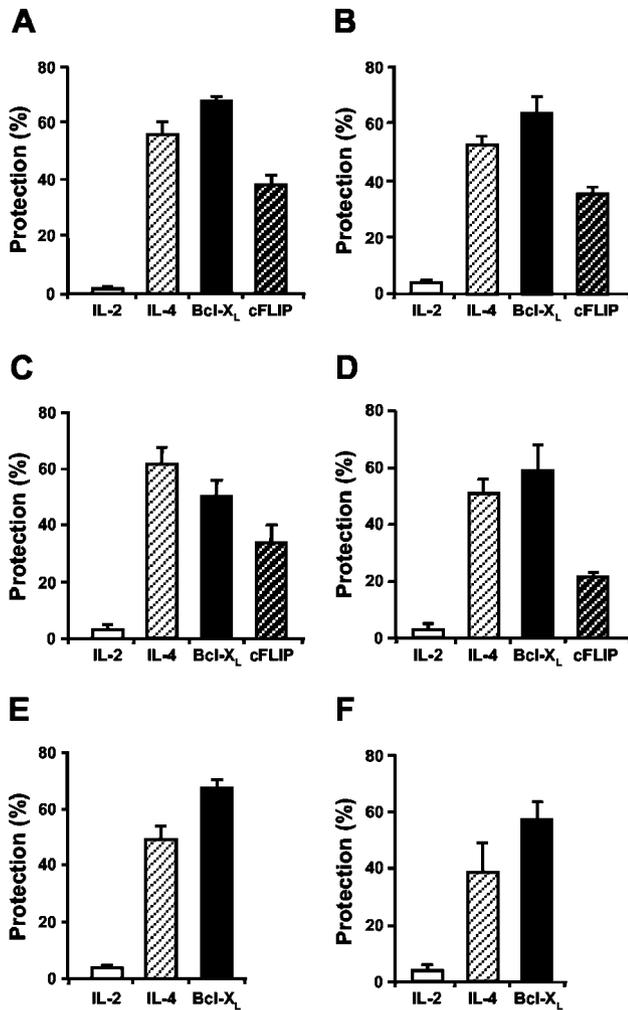


FIGURE 4. Exogenous expression of Bcl-x_L or cFLIP/FLAME-1 prevents chemotherapy-induced apoptosis in tumor cells. Cell lines stably overexpressing Bcl-x_L or cFLIP/FLAME-1 were treated for 24 h with chemotherapeutic drugs. RT112 cells were treated with camptothecin (A) or etoposide (B), LNCaP cells treated with cisplatin (C) or vincristine (D), and MDA-MB-231 cells treated with cisplatin (E) or daunorubicin (F). The results shown are the mean ± SD of five independent experiments.

the CD95 DISC by blocking the activation of caspase-8 (6), whereas overexpression of Bcl-x_L inhibits the mitochondrial apoptotic pathways, which contribute to the execution phase of death receptor signaling (36). To determine the capacity of Bcl-x_L and cFLIP/FLAME-1 to inhibit CD95-induced apoptosis of tumor cells, RT112, MDA-MB-231, and LNCaP cells stably overexpressing Bcl-x_L, and RT112 and LNCaP cells overexpressing cFLIP/FLAME-1 were stimulated with anti-CD95 agonistic Abs. Bcl-x_L was able to induce a significant protection of the three cell lines from CD95-mediated death, indicating an important role of mitochondrial events in CD95-mediated apoptosis of these cells (Fig. 5), while cFLIP/FLAME-1 efficiently protected LNCaP and RT112 cells from CD95-induced apoptosis at levels comparable to those obtained with IL-4 treatment (Fig. 5, A and B). These results support the hypothesis that the up-regulation of Bcl-x_L and cFLIP/FLAME-1 induced by IL-4 may contribute to an increased resistance of tumors to apoptosis mediated by chemotherapeutic drugs and by immune system effectors.

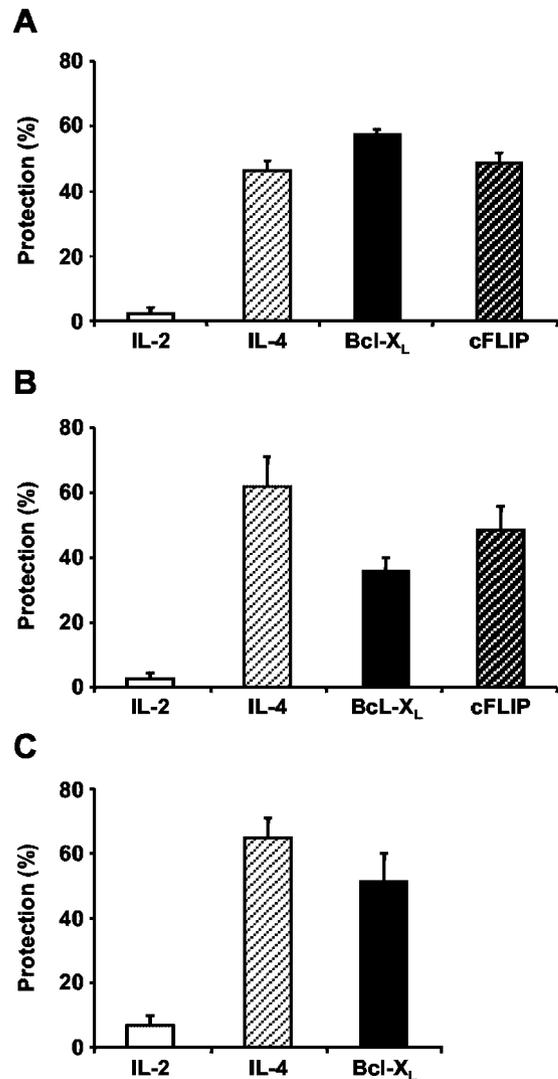


FIGURE 5. Exogenous expression of Bcl-x_L or cFLIP/FLAME-1 protects tumor cells from anti-CD95-induced apoptosis. RT112 (A), LNCaP (B), and MDA-MB-231 (C) cell lines stably overexpressing Bcl-x_L or cFLIP/FLAME-1 were treated for 24 h with anti-CD95 agonistic Ab. The results shown are the mean ± SD of five independent experiments.

In vivo production of IL-4 is associated with up-regulation of Bcl-x_L and cFLIP/FLAME-1 in bladder and prostate cancer

To determine the role of IL-4 in tumor cell protection *in vivo*, we analyzed bladder, prostate, and breast cancer specimens by immunohistochemistry to detect the presence of IL-4 and the expression of IL-4-induced antiapoptotic proteins. In line with literature data, normal and neoplastic tissues consistently showed the presence of IL-4 in all the different types of cancer examined, while normal tissues were essentially negative (Fig. 6). Serial section analysis indicated that IL-4 reactivity was associated with the presence of a CD45⁺ immune infiltrate, which most likely represents the major source of IL-4 production in the different tumors (Fig. 6, A–C). Analysis of apoptosis-related proteins showed that bladder, prostate, and breast tumors display high reactivity for both Bcl-x_L and cFLIP/FLAME-1, whose expression in normal tissues was extremely low or undetectable (Fig. 7A). As expected, the three types of tumor expressed the IL-4R, which was also present in normal tissues (Fig. 7A). Moreover, we compared the levels of Bcl-x_L and cFLIP/FLAME-1 observed *in vivo* with those of cells lines whose

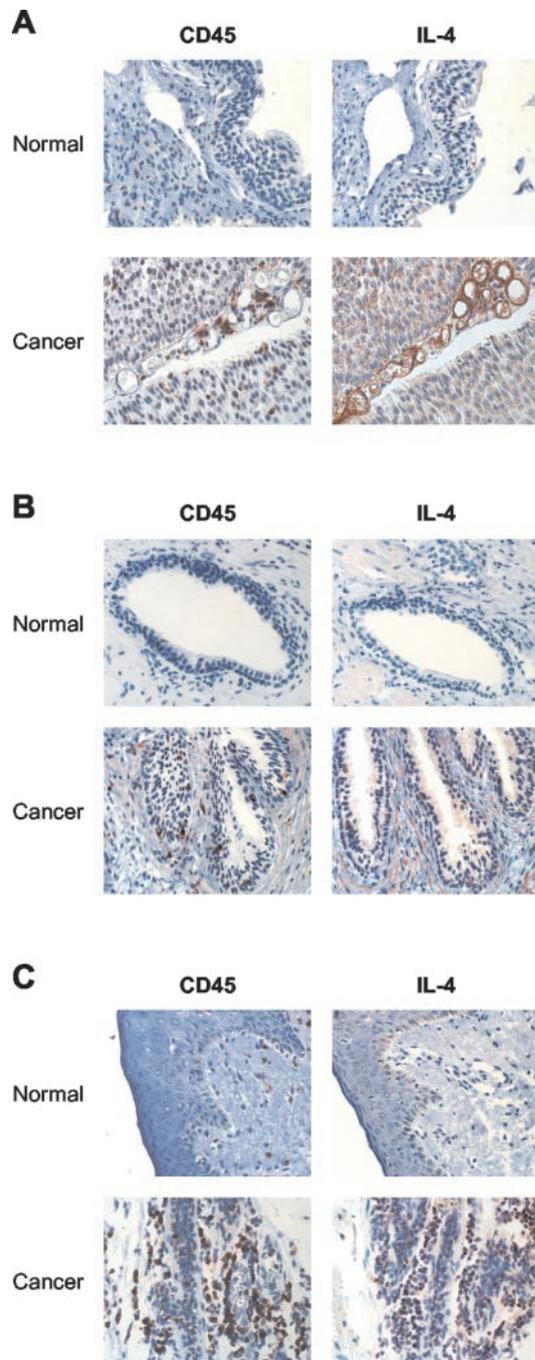


FIGURE 6. Production of IL-4 in bladder, prostate, and breast cancer. Immunohistochemical analysis of paraffin-embedded normal or bladder (A), prostate (B), and breast (C) cancer sections labeled with anti-CD45 or anti-IL-4 and revealed by 3-amino-9-ethylcarbazole (red staining).

exogenous expression of either Bcl-x_L or cFLIP/FLAME-1 conferred protection from chemotherapeutic drugs and anti-CD95. The expression of both Bcl-x_L and cFLIP/FLAME-1 was similar or even higher in bladder and prostate cancer as compared with the corresponding cell lines carrying the exogenous gene (Fig. 7, B and C), while the *in vivo* expression of Bcl-x_L in breast cancer was slightly lower than that found in transduced MDA-MB-231 (Fig. 7D). Thus, *in vivo* production of IL-4 in bladder and prostate cancer is associated with high expression of Bcl-x_L and cFLIP/FLAME-1, which protect cancer cells from chemotherapy and CD95-mediated apoptosis.

In vivo production of IL-4 protects breast cancer cells from chemotherapy

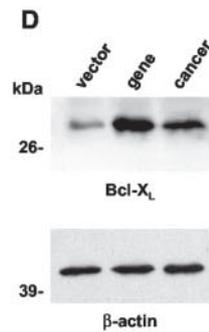
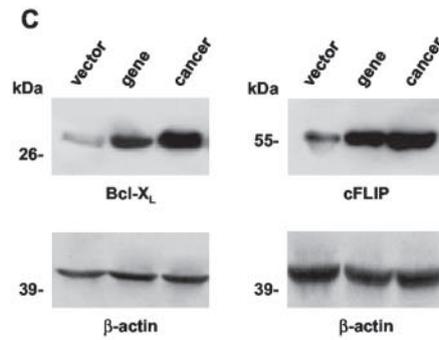
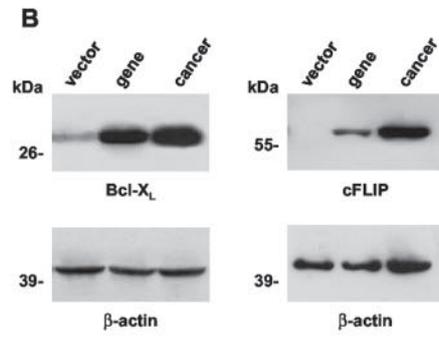
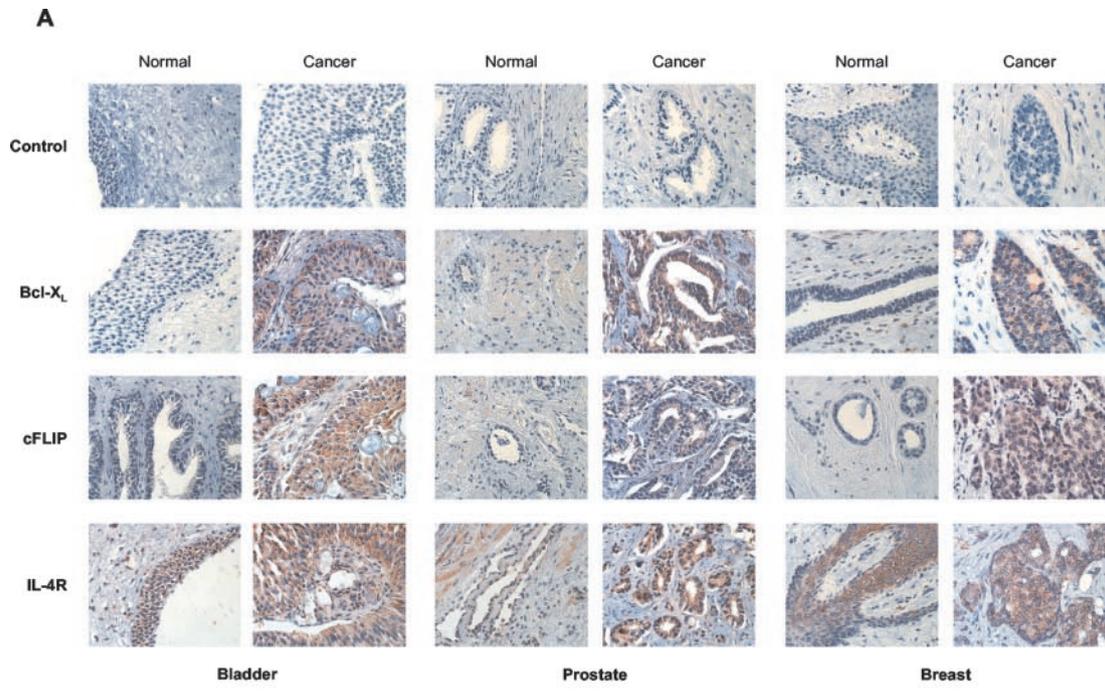
To confirm that IL-4-induced up-regulation of Bcl-x_L is able to protect epithelial breast cells from apoptosis, we exposed freshly isolated normal breast epithelial cells to IL-4 and analyzed Bcl-x_L expression and sensitivity to a panel of chemotherapeutic drugs commonly used to treat breast cancer. IL-4 treatment of normal breast epithelial cells considerably increased the expression of Bcl-x_L, which reached levels slightly lower than those observed in freshly isolated neoplastic cells (Fig. 8A). Accordingly, IL-4 significantly protected normal breast epithelial cells from apoptosis induced by cisplatin, doxorubicin, and taxol (Fig. 8B). To confirm that *in vivo* exposure to IL-4 is responsible for high Bcl-x_L expression and resistance to chemotherapy in breast cancer, we measured Bcl-x_L expression and sensitivity to apoptosis of primary breast carcinoma cells cultivated in the presence or absence of IL-4. Freshly isolated breast cancer cells expressed high levels of Bcl-x_L and were scarcely sensitive to chemotherapeutic drugs (Fig. 8, C and D). However, after 6 days of *in vitro* culture in the absence of IL-4, breast cancer cells down-regulated Bcl-x_L and became sensitive to chemotherapy-induced apoptosis, while in the presence of IL-4 they maintained high Bcl-x_L levels and low sensitivity to chemotherapeutic drugs (Fig. 8, C and D). Thus, *in vivo* IL-4 production promotes the survival of breast cancer cells.

Discussion

The production of cytokines by TILs within the tumor microenvironment plays a key role in the development of an immune response against neoplastic cells. Th1-type cytokines support the activation and stimulation of CTL, which act predominantly as immune effectors by inducing apoptotic death in target cells (37, 38). Conversely, Th2-type cytokines are associated with increased susceptibility to tumor challenge in animal models (39) and have been proposed to induce immunosuppression at the tumor site by inhibiting IL-2-mediated T cell activation as well as the production of monocyte-inflammatory cytokines (40–42).

A gradual loss of Th1 lymphocyte population and an increase of Th2 cytokine profile have been reported to occur during progressive tumor growth in murine models of renal cell carcinoma and colon adenocarcinoma (38), suggesting that a shift from Th1 to Th2 response may be important for the development of cancer. Moreover, a decreased Th1/Th2 ratio has been described in a plethora of human malignancies, including glioblastoma (43), lung cancer (44), breast cancer (45), urinary, bladder, renal cell, and prostate cancer (22), indicating the existence of a local and peripheral Th2-type cytokine pattern in the majority of cancer patients.

Cytokines produced by Th2 lymphocytes have been proposed to promote cell survival by influencing the expression of proteins involved in the regulation of apoptosis. Expansion of a peculiar Th2 lymphocyte subset with increased IL-4 production has been found in patients with B cell chronic lymphocytic leukemia (46). Because IL-4 induces Bcl-2 expression in B cell chronic lymphocytic leukemia cells and inhibits spontaneous and hydrocortisone-induced apoptosis, it has been suggested that IL-4 prevents death of malignant B cells through a Bcl-2-dependent pathway (19). The differential production of Th1 and Th2 cytokines has also been reported to influence cell survival in organ-specific autoimmune diseases. In two opposite thyroid autoimmune conditions, Hashimoto's thyroiditis and Graves' disease, Th1 and Th2 cytokines have been shown to modulate the levels of apoptosis-related proteins. Specifically, IL-4 produced by thyroid-infiltrating Th2 lymphocytes has been demonstrated to potently up-regulate Bcl-x_L and



cFLIP/FLAME-1 in autoimmune thyrocytes of Graves' disease patients, thus representing the likely cause of thyrocyte resistance to CD95-mediated apoptosis and possibly contributing to hyperthyroidism (20). Moreover, autocrine production of IL-4 and IL-10 has been shown recently to protect thyroid cancer cells from chemotherapy (47). Given the possibility that cytokines produced by type 2 TILs may promote tumor progression through the induction of apoptosis-resistant features in neoplastic cells, we investigated the prosurvival effects of IL-4 on cells derived from solid tumors. This study shows that IL-4 strongly inhibits apoptosis induced by CD95 and chemotherapeutic drugs in cells derived from bladder, prostate, and breast carcinomas through the up-regulation of Bcl-x_L and cFLIP/FLAME-1.

Tumor cells have been previously demonstrated to evade death signals generated by immune effectors or by therapeutic drugs through the development of effective antiapoptotic mechanism such as increased levels of caspase inhibitors or Bcl-2-family members (4). Overexpression of cFLIP/FLAME-1 or viral FLIP has been described in melanomas (13), lymphomas (48), and late-stage Kaposi sarcomas (49), suggesting the existence of a causal relationship between high FLIP/FLAME-1 expression and tumorigenesis (14). A dysregulation of Bcl-2 and/or Bcl-x_L has been reported in several human cancers (4) and has been shown to contribute to the resistance of neoplastic cells to the action of chemotherapeutic drugs (10, 31–33). Therefore, the up-regulation of Bcl-x_L and cFLIP/FLAME-1 induced by IL-4 in tumor cells may provide an explanation for IL-4-mediated protection of these cells from apoptosis.

Interestingly, we observed that IL-4-mediated up-regulation of Bcl-x_L and cFlip/FLAME-1 in tumor cells does not correlate with increased production of the corresponding mRNA (data not shown). A similar finding has been recently reported for fibroblast growth factor-2, which prevents etoposide-induced apoptosis by up-regulating Bcl-2 and Bcl-x_L protein levels through a translational mechanism dependent on activation of mitogen-activated protein (MAP)/extracellular signal-regulated kinase kinases (50). Because IL-4R α activates insulin receptor substrate-1 and -2 (51), which in turn mediate activation of the Ras/MAP pathway (52), it may be speculated that a MAP kinase-mediated mechanism may be responsible for Bcl-x_L and cFlip/FLAME-1 up-regulation induced by IL-4 in tumor cells.

Since the first observations that solid human tumors express high affinity IL-4Rs (53, 54) and that IL-4 is able to inhibit the growth of several tumor cell types in vitro (55–57), IL-4 effects on neoplastic cells have been extensively studied in the attempt to find a rationale for a therapeutic use of this cytokine in the treatment of human cancer. IL-4 is required for the development of effective tumor immunity (58). Moreover, an indirect antitumor activity of IL-4 in vivo, mediated by T cells, macrophages, and eosinophils, has been detected following injection of IL-4-transfected tumor cells in mice (59, 60), supporting a potential use of IL-4 in tumor therapy. However, administration of IL-4 for the treatment of both hematologic and nonhematologic cancers has produced unsatisfactory results (61–64), yielding minor or negligible effects or even leading to a possible increase in the number of malignant cells (61).

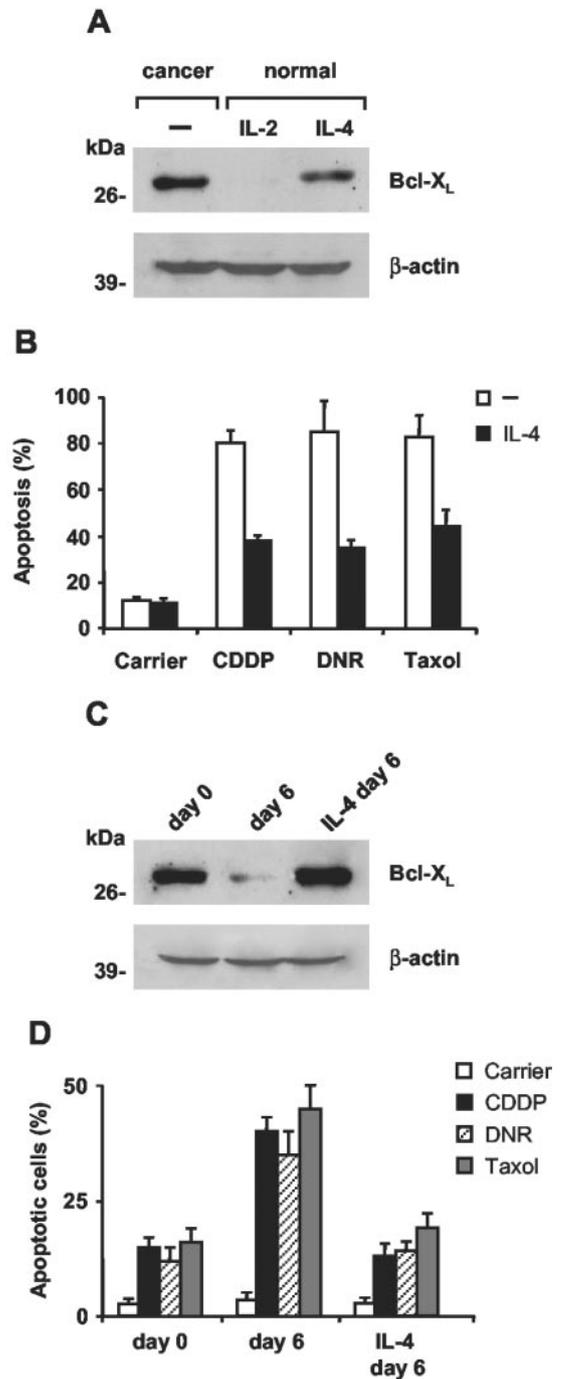


FIGURE 8. IL-4 protects primary breast cancer cells from apoptosis. *A*, Immunoblot analysis of Bcl-x_L expression in freshly isolated primary breast epithelial cancer or normal cells (–) and in normal primary breast epithelial treated for 2 days with 20 ng/ml IL-4. *B*, Percentage of cell death in normal primary breast epithelial treated as described in *A* and exposed to cisplatin (CDDP), daunorubicin (DNR), or taxol. Control cells were stimulated with chemotherapeutic drugs in the absence of cytokine pretreatment. *C*, Immunoblot analysis of Bcl-x_L expression in primary cancer cells freshly isolated (day 0) or cultivated for 6 days in the absence (day 6) or in the presence of 20 ng/ml IL-4 (IL-4 day 6). *D*, Percentage of apoptotic cell death in primary tumor cells treated as in *C* and exposed for 24 h to cisplatin, daunorubicin, or taxol.

FIGURE 7. Bladder, prostate, and breast cancer express high levels of Bcl-x_L and cFLIP/FLAME-1. *A*, Immunohistochemical analysis of Bcl-x_L, cFLIP/FLAME-1, or IL-4R on paraffin sections of normal or bladder, prostate, and breast cancer specimens (red). Expression of Bcl-x_L and cFLIP/FLAME-1 in freshly isolated primary tumor cells (cancer) from bladder (*B*), prostate (*C*), and breast (*D*) carcinomas as compared with RT112 (*B*), LNCaP (*C*), and MDA-MB-231 (*D*) cancer cell lines retrovirally transduced with the empty vector (vector) or with the relevant gene (gene). One representative of three independent experiments is shown.

Data presented in this study show the ability of IL-4 to up-modulate antiapoptotic proteins in cancer cells, and thus may represent a caveat for the use of this cytokine in anticancer therapy. Elucidation of the complex interactions between host immune effectors and neoplastic cells is fundamental to the understanding of the immunological response to cancer and may lead to the identification of new targets for therapeutic intervention.

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References

- Blagosklonny, M. V., and A. B. Pardee. 2001. Exploiting cancer cell cycling for selective protection of normal cells. *Cancer Res.* 61:4301.
- Strand, S., and P. R. Galle. 1998. Immune evasion by tumors: involvement of the CD95 (APO-1/Fas) system and its clinical implications. *Mol. Med. Today* 4:63.
- Herr, I., and K. M. Debatin. 2001. Cellular stress response and apoptosis in cancer therapy. *Blood* 98:2603.
- Reed, J. C. 1999. Dysregulation of apoptosis in cancer. *J. Clin. Oncol.* 17:2941.
- Peter, M. E., and P. H. Krammer. 1998. Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis. *Curr. Opin. Immunol.* 10:545.
- Scaffidi, C., I. Schmitz, P. H. Krammer, and M. E. Peter. 1999. The role of c-FLIP in modulation of CD95-induced apoptosis. *J. Biol. Chem.* 274:1541.
- Krueger, A., S. Baumann, P. H. Krammer, and S. Kirchhoff. 2001. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Mol. Cell. Biol.* 21:8247.
- Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri, and X. Wang. 1997. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91:479.
- Green, D. R., and J. C. Reed. 1998. Mitochondria and apoptosis. *Science* 281:1309.
- Yang, J., X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. I. Peng, D. P. Jones, and X. Wang. 1997. Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science* 275:1129.
- Adams, J. M., and S. Cory. 1998. The Bcl-2 protein family: arbiters of cell survival. *Science* 281:1322.
- Reed, J. C. 1997. Double identity for proteins of the Bcl-2 family. *Nature* 387:773.
- Medema, J. P., J. de Jong, T. van Hall, C. J. Melief, and R. Offringa. 1999. Immune escape of tumors in vivo by expression of cellular FLICE-inhibitory protein. *J. Exp. Med.* 190:1033.
- Djerbi, M., V. Screpanti, A. I. Catrina, B. Bogen, P. Biberfeld, and A. Grandien. 1999. The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. *J. Exp. Med.* 190:1025.
- Mukherji, B., N. G. Chakraborty, and M. Sivanandham. 1990. T-cell clones that react against autologous human tumors. *Immunol. Rev.* 116:33.
- Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature* 383:787.
- Shurin, M. R., L. Lu, P. Kalinski, A. M. Stewart-Akers, and M. T. Lotze. 1999. Th1/Th2 balance in cancer, transplantation and pregnancy. *Springer Semin. Immunopathol.* 21:339.
- Stassi, G., and R. De Maria. 2002. Autoimmune thyroid disease: new models of cell death in autoimmunity. *Nat. Rev. Immunol.* 2:195.
- Dancescu, M., M. Rubio-Trujillo, G. Biron, D. Bron, G. Delespesse, and M. Sarfati. 1992. Interleukin 4 protects chronic lymphocytic leukemic B cells from death by apoptosis and up-regulates Bcl-2 expression. *J. Exp. Med.* 176:1319.
- Stassi, G., D. Di Liberto, M. Todaro, A. Zeuner, L. Ricci-Vitiani, A. Stoppacciaro, L. Ruco, F. Farina, G. Zummo, and R. De Maria. 2000. Control of target cell survival in thyroid autoimmunity by T helper cytokines via regulation of apoptotic proteins. *Nat. Immunol.* 1:483.
- Wise, G. J., V. K. Marella, G. Talluri, and D. Shirazian. 2000. Cytokine variations in patients with hormone treated prostate cancer. *J. Urol.* 164:722.
- Elsasser-Beile, U., N. Kolble, T. Grussenmeyer, W. Schultze-Seemann, U. Wetterauer, H. Gallati, J. Schulte Monting, and S. von Kleist. 1998. Th1 and Th2 cytokine response patterns in leukocyte cultures of patients with urinary bladder, renal cell and prostate carcinomas. *Tumour Biol.* 19:470.
- Schondorf, T., H. Engel, C. Lindemann, H. Kolhagen, A. A. von Rucker, and P. Mallmann. 1997. Cellular characteristics of peripheral blood lymphocytes and tumor-infiltrating lymphocytes in patients with gynecological tumors. *Cancer Immunol. Immunother.* 44:88.
- Camp, B. J., S. T. Dyhrman, V. A. Memoli, L. A. Mott, and R. J. Barth, Jr. 1996. In situ cytokine production by breast cancer tumor-infiltrating lymphocytes. *Ann. Surg. Oncol.* 3:176.
- De Maria, R., A. Zeuner, A. Eramo, C. Domenichelli, D. Bonci, F. Grignani, S. M. Srinivasula, E. S. Alnemri, U. Testa, and C. Peschle. 1999. Negative regulation of erythropoiesis by caspase-mediated cleavage of GATA-1. *Nature* 401:489.
- Kobayashi, M., H. Kobayashi, R. B. Pollard, and F. Suzuki. 1998. A pathogenic role of Th2 cells and their cytokine products on the pulmonary metastasis of murine B16 melanoma. *J. Immunol.* 160:5869.
- Pinkoski, M. J., and D. R. Green. 1999. Fas ligand, death gene. *Cell Death Differ.* 6:1174.
- Gronbaek, K., P. T. Straten, E. Ralfkiaer, V. Ahrenkiel, M. K. Andersen, N. E. Hansen, J. Zeuthen, K. Hou-Jensen, and P. Guldberg. 1998. Somatic Fas mutations in non-Hodgkin's lymphoma: association with extranodal disease and autoimmunity. *Blood* 92:3018.
- Hahne, M., D. Rimoldi, M. Schroter, P. Romero, M. Schreier, L. E. French, P. Schneider, T. Bornand, A. Fontana, D. Lienard, et al. 1996. Melanoma cell expression of Fas (Apo-1/CD95) ligand: implications for tumor immune escape. *Science* 274:1363.
- Pitti, R. M., S. A. Marsters, D. A. Lawrence, M. Roy, F. C. Kischkel, P. Dowd, A. Huang, C. J. Donahue, S. W. Sherwood, D. T. Baldwin, et al. 1998. Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* 396:699.
- Minn, A. J., C. M. Rudin, L. H. Boise, and C. B. Thompson. 1995. Expression of Bcl-x_L can confer a multidrug resistance phenotype. *Blood* 86:1903.
- Campos, L., J. P. Rouault, O. Sabido, P. Oriol, N. Roubi, C. Vasselon, E. Archimbaud, J. P. Magaud, and D. Guyotat. 1993. High expression of *bcl-2* protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood* 81:3091.
- Ibrado, A. M., Y. Huang, G. Fang, L. Liu, and K. Bhalla. 1996. Overexpression of Bcl-2 or Bcl-x_L inhibits Ara-C-induced CPP32/Yama protease activity and apoptosis of human acute myelogenous leukemia HL-60 cells. *Cancer Res.* 56:4743.
- Kataoka, T., R. C. Budd, N. Holler, M. Thome, F. Martinon, M. Irmeler, K. Burns, M. Hahne, N. Kennedy, M. Kovacsovic, and J. Tschopp. 2000. The caspase-8 inhibitor FLIP promotes activation of NF- κ B and Erk signaling pathways. *Curr. Biol.* 10:640.
- Fulda, S., M. Los, C. Friesen, and K. M. Debatin. 1998. Chemosensitivity of solid tumor cells in vitro is related to activation of the CD95 system. *Int. J. Cancer* 76:105.
- Scaffidi, C., S. Fulda, A. Srinivasan, C. Friesen, F. Li, K. J. Tomaselli, K. M. Debatin, P. H. Krammer, and M. E. Peter. 1998. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.* 17:1675.
- Matsui, S., J. D. Ahlers, A. O. Vortmeyer, M. Terabe, T. Tsukui, D. P. Carbone, L. A. Liotta, and J. A. Berzofsky. 1999. A model for CD8⁺ CTL tumor immunosurveillance and regulation of tumor escape by CD4 T cells through an effect on quality of CTL. *J. Immunol.* 163:184.
- Ghosh, P., K. L. Komschlies, M. Cippitelli, D. L. Longo, J. Subleski, J. Ye, A. Sica, H. A. Young, R. H. Wiltrout, and A. C. Ochoa. 1995. Gradual loss of T-helper 1 populations in spleen of mice during progressive tumor growth. *J. Natl. Cancer Inst.* 87:1478.
- Lee, P. P., D. Zeng, A. E. McCaulay, Y. F. Chen, C. Geiler, D. T. Umetsu, and N. J. Chao. 1997. T helper 2-dominant antilymphoma immune response is associated with fatal outcome. *Blood* 90:1611.
- Yamamura, M., R. L. Modlin, J. D. Ohmen, and R. L. Moy. 1993. Local expression of antiinflammatory cytokines in cancer. *J. Clin. Invest.* 91:1005.
- Martinez, O. M., R. S. Gibbons, M. R. Garovoy, and F. R. Aronson. 1990. IL-4 inhibits IL-2 receptor expression and IL-2-dependent proliferation of human T cells. *J. Immunol.* 144:2211.
- Hart, P. H., G. F. Vitti, D. R. Burgess, G. A. Whitty, D. S. Piccoli, and J. A. Hamilton. 1989. Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor α , interleukin 1, and prostaglandin E₂. *Proc. Natl. Acad. Sci. USA* 86:3803.
- Ashkenazi, E., M. Deutsch, R. Tirosh, A. Weinreb, A. Tsukerman, and C. Brodie. 1997. A selective impairment of the IL-2 system in lymphocytes of patients with glioblastomas: increased level of soluble IL-2R and reduced protein tyrosine phosphorylation. *Neuroimmunomodulation* 4:49.
- Chen, Y. M., W. K. Yang, C. C. Ting, W. Y. Tsai, D. M. Yang, J. Whang-Peng, and R. P. Perng. 1997. Cross regulation by IL-10 and IL-2/IL-12 of the helper T cells and the cytolytic activity of lymphocytes from malignant effusions of lung cancer patients. *Chest* 112:960.
- Rosen, H. R., C. Ausch, M. Reinerova, E. Zaspin, K. Renner, A. C. Rosen, R. Schiessel, and C. Moroz. 1998. Activated lymphocytes from breast cancer patients express the characteristics of type 2 helper cells: a possible role for breast cancer-associated p43. *Cancer Lett.* 127:129.
- De Toter, D., G. Reato, F. Mauro, A. Cignetti, S. Ferrini, A. Guarini, M. Gobbi, C. E. Grossi, and R. Foa. 1999. IL4 production and increased CD30 expression by a unique CD8⁺ T-cell subset in B-cell chronic lymphocytic leukemia. *Br. J. Haematol.* 104:589.
- Stassi, G., M. Todaro, M. Zerilli, L. Ricci-Vitiani, D. Di Liberto, M. Patti, A. Florena, F. Di Gaudio, G. Di Gesu, and R. De Maria. 2003. Thyroid cancer resistance to chemotherapeutic drugs via autocrine production of interleukin-4 and interleukin-10. *Cancer Res.* 63:6784.
- Sturzl, M., C. Hohenadl, C. Zietz, E. Castanos-Velez, A. Wunderlich, G. Ascherl, P. Biberfeld, P. Monini, P. J. Browning, and B. Ensoli. 1999. Expression of K13/v-FLIP gene of human herpesvirus 8 and apoptosis in Kaposi's sarcoma spindle cells. *J. Natl. Cancer Inst.* 91:1725.
- Low, W., M. Harries, H. Ye, M. Q. Du, C. Boshoff, and M. Collins. 2001. Internal ribosome entry site regulates translation of Kaposi's sarcoma-associated herpesvirus FLICE inhibitory protein. *J. Virol.* 75:2938.
- Pardo, O. E., A. Arcaro, G. Salerno, S. Raguz, J. Downward, and M. J. Seckl. 2002. Fibroblast growth factor-2 induces translational regulation of Bcl-x_L and Bcl-2 via a MEK-dependent pathway: correlation with resistance to etoposide-induced apoptosis. *J. Biol. Chem.* 277:12040.

51. Keegan, A. D., K. Nelms, M. White, L. M. Wang, J. H. Pierce, and W. E. Paul. 1994. An IL-4 receptor region containing an insulin receptor motif is important for IL-4-mediated IRS-1 phosphorylation and cell growth. *Cell* 76:811.
52. Jiang, H., M. B. Harris, and P. Rothman. 2000. IL-4/IL-13 signaling beyond JAK/STAT. *J. Allergy Clin. Immunol.* 105:1063.
53. Obiri, N. I., J. P. Siegel, F. Varricchio, and R. K. Puri. 1994. Expression of high-affinity IL-4 receptors on human melanoma, ovarian and breast carcinoma cells. *Clin. Exp. Immunol.* 95:148.
54. Al Jabaari, B., H. M. Ladyman, M. Larche, G. B. Sivolapenko, A. A. Epenetos, and M. A. Ritter. 1989. Elevated expression of the interleukin 4 receptor in carcinoma: a target for immunotherapy? *Br. J. Cancer* 59:910.
55. Obiri, N. I., G. G. Hillman, G. P. Haas, S. Sud, and R. K. Puri. 1993. Expression of high affinity interleukin-4 receptors on human renal cell carcinoma cells and inhibition of tumor cell growth in vitro by interleukin-4. *J. Clin. Invest.* 91:88.
56. Hoon, D. S., E. Okun, M. Banez, R. F. Irie, and D. L. Morton. 1991. Interleukin 4 alone and with γ -interferon or α -tumor necrosis factor inhibits cell growth and modulates cell surface antigens on human renal cell carcinomas. *Cancer Res.* 51:5687.
57. Toi, M., R. Bicknell, and A. L. Harris. 1992. Inhibition of colon and breast carcinoma cell growth by interleukin-4. *Cancer Res.* 52:275.
58. Schuler, T., Z. Qin, S. Ibe, N. Noben-Trauth, and T. Blankenstein. 1999. T helper cell type 1-associated and cytotoxic T lymphocyte-mediated tumor immunity is impaired in interleukin 4-deficient mice. *J. Exp. Med.* 189:803.
59. Tepper, R. I., P. K. Pattengale, and P. Leder. 1989. Murine interleukin-4 displays potent anti-tumor activity in vivo. *Cell* 57:503.
60. Golumbek, P. T., A. J. Lazenby, H. I. Levitsky, L. M. Jaffee, H. Karasuyama, M. Baker, and D. M. Pardoll. 1991. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science* 254:713.
61. Lundin, J., E. Kimby, L. Bergmann, T. Karakas, H. Mellstedt, and A. Osterborg. 2001. Interleukin 4 therapy for patients with chronic lymphocytic leukemia: a phase I/II study. *Br. J. Haematol.* 112:155.
62. Taylor, C. W., M. LeBlanc, R. I. Fisher, D. F. Moore, Sr., R. W. Roach, L. Elias, and T. P. Miller. 2000. Phase II evaluation of interleukin-4 in patients with non-Hodgkin's lymphoma: a Southwest Oncology Group trial. *Anticancer Drugs* 11:695.
63. Whitehead, R. P., J. M. Unger, J. W. Goodwin, M. J. Walker, J. A. Thompson, L. E. Flaherty, and V. K. Sondak. 1998. Phase II trial of recombinant human interleukin-4 in patients with disseminated malignant melanoma: a Southwest Oncology Group study. *J. Immunother.* 21:440.
64. Stadler, W. M., M. E. Rybak, and N. J. Vogelzang. 1995. A phase II study of subcutaneous recombinant human interleukin-4 in metastatic renal cell carcinoma. *Cancer* 76:1629.