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Attack the Tumor Counterattack-C-Flip Expression in Jurkat-T-Cells Protects Against Apoptosis Induced by Coculture with SW620 Colorectal Adenocarcinoma Cells

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Background. **Cancer development relies on a variety of mechanisms that facilitate tumor growth despite the presence of a functioning immune system, employing different mechanisms to escape immune rejection. Tumors may eliminate tumor-infiltrating lymphocytes and suppress anti-tumor immune responses, a process called "tumor counterattack," based on activation-induced cell death *via* the FAS/FAS-ligand system. To overcome this tumor-cell survival strategy, we examined the hypothesis that the sensitivity of FAS mediated apoptosis of Jurkat-T-cells can be suppressed by FLIP transfection of Jurkat-T-cells.**

Materials and Methods. **Jurkat-T-cells were transfected with the FLICE-inhibitory protein FLIP in order to bestow them with a resistance to FAS-receptor-mediated apoptosis. FLIP-transfected and non-transfected Jurkat-T-cells were grown in coincubation with SW620 cells and the rates of apoptosis measured *via* FACS-analysis of Annexin-V.**

Results. **First, the tumor-counterattack described in the literature was confirmed. About 20% of Jurkat-T-Cells underwent apoptosis in coculture with SW620 cells. After coincubation of SW620 cells with FLIP transfected Jurkat-T-cells the apoptotic rate was significant reduced at levels below 4%.**

Conclusion. **Transfection of Jurkat-T-cells with FLIP reduces the sensitivity of Jurkat-T-cells to FAS-mediated apoptosis and may lead to an improved capability to antagonize the inherent tumor survival strategy of SW620 cells.**

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Key Words: **apoptosis; colon carcinoma; tumor counterattack; immunotherapy; gene therapy; FLICE-inhibitory-protein (FLIP).**

INTRODUCTION

The establishment and progression of malignant cells to the formation of a tumor is dependent on several mechanisms. An intact immune system is capable of detecting tumor cells and destroying them through cell-to-cell or cytotoxic mechanisms [1–5]. The so-called tumor-infiltrating lymphocytes (TIL) migrate to and then enter the tumor; these represent the most important, specific immune response against the tumor [3]. Various subpopulations of lymphocytes can be detected in tumor samples, including CD8+, CD4+, natural killer cells (NK), macrophages, and dendritic cells (DCs) [6–10].

During tumorigenesis, tumor cell populations first acquire properties enabling their local invasion into surrounding organ structures as well as their hematogenic and lymphatic metastasis [3, 11]. Possible explanations for how tumor cells can survive and multiply as a quantitative minority among differentiated cell populations in the lymphatic and circulatory systems represent so-called "escape strategies," which are developed by a wide variety of tumors during tumorigenesis [3, 12–15].

One of the recognized tumor escape strategies lies in altering of the expression of MHC molecules. Many known tumor types only express MHC Class I molecules and not Class II. This allows them to escape recognition by Th-2 helper cells (Th2) [6, 7]. Research into auto-immune diseases has described the FAS/FAS-ligand system, which

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plays a key role in the lymphocytic homeostasis of the immune response [16, 17]. To avoid an excessive immune response, activated lymphocytes increase the expression and sensitivity of their FAS receptors, binding to which begins the process of apoptosis in the cells. This autoregulatory suppression of the immune response is mediated by FAS-ligand-expressing CD8+ and Th1 cells [5]. It is exactly this mechanism of autoregulatory immune suppression that could represent a survival strategy for tumor cells.

Various tumor entities express FAS-ligand in high levels and are not themselves sensitive to FAS-mediated apoptosis [13–15, 18–22]. As such, tumor cells that express FAS ligand stimulate apoptosis in tumor-infiltrating lymphocytes, thereby removing one of the immune system's mechanisms for the destruction of tumor cells.

During the search for regulatory mechanisms of the FAS/FAS-ligand system, Thome *et al.* described a new family of proteins, FLIP, (FLICE-inhibitory protein [FLICE ≈ FADD-like interleukin-1ß-converting-enzyme]) [23]. vFLIP proteins are viral inhibitors and are found in various herpes viruses including Karposi's-sarcoma-associated human herpes virus 8 and the human molluscipox virus [23–25]. The protein $FLIP_S$ contains two death effector domains (DED), which can interact with the FAS-associated death domain (FADD) and caspase-8 and possibly caspase-10.

The binding of FAS-ligand on the FAS receptor leads to a new re-formation of these transmembrane and intracellular proteins to a death-inducing signaling complex (DISC) [26–28]. An overexpression of FLIP leads to the inclusion of FLIP in the DISC. This inclusion blocks the intracellular cascade, leading to activation-induced cell death (AICD) [5, 29–31], whereby the FLIP-mediated activation of caspase-8 inhibits the AICD [32] (Fig. 1).

Moreover, the higher the expression of FLIP and its integration into the DISC are, the higher the resistance of the cell to apoptosis. This may involve a competitive effect of caspase-8 and FLIP for integration into DISC. $FLIP_S$ and $FLIP_L$ are able to inhibit apoptosis *via* all known death receptors, FAS, TRAMP (wsl/DR-3/APO-3), TRAIL-R (DR-4), and tumor necrosis factor receptor 1 (TNF-R1) [32].

In summary, c-FLIP is an anti-apoptotic molecule for which a high level of expression quantitatively correlates to resistance to apoptosis [33].

The innovative approach of this study lies in inhibiting the FAS-ligand-expressing tumor cells' ability to eliminate FAS-sensitive T-cells, thereby disrupting the tumor's escape strategy. To that end, Jurkat-T-cells were transfected with FLIP and their rates of apoptosis in coincubation with SW620 colorectal adenocarcinoma cells examined.

MATERIALS AND METHODS

Cell Culture

Jurkat T-cells are consistent with acute T-cell leukaemia cells and appear morphologically identical to lymphoblasts; Jurkat T-cells (American Type Culture Collection (ATCC), Manassas, VA, order number: CRL-2063). SW620 colorectal adenocarcinoma cells were isolated from a lymph node metastasis of a 51-y-old Caucasian as was

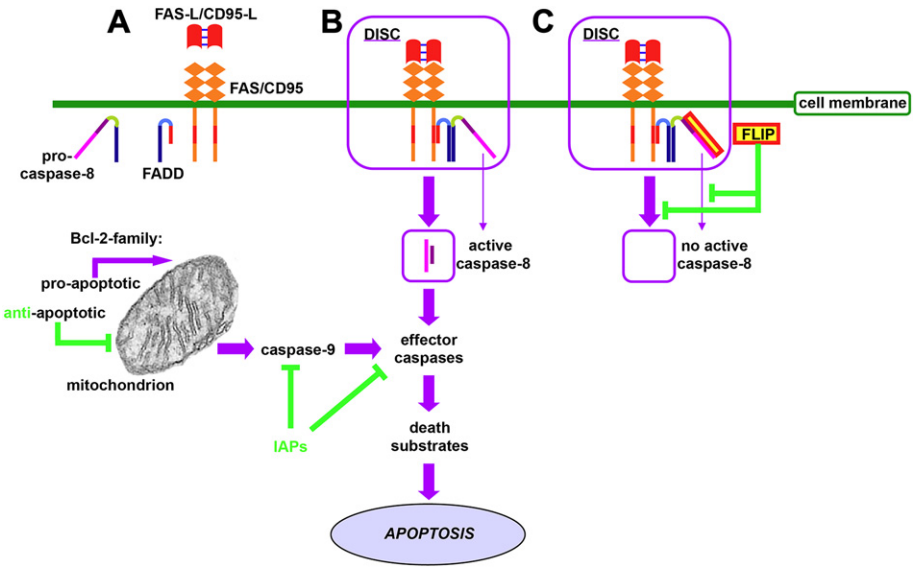


FIG. 1. Apoptosis signaling *via* the Fas/Fas-Ligand pathway. (A) Shows the different proteins FAS, FAS-Ligand, FADD, and pro-caspase-8 that are involved to create the death inducing signaling complex (DISC). (B) Shows the binding of FAS-ligand on the FAS receptor leading to DISC formation and thereby activation of pro-caspase-8 into active caspase 8, which is the key protein in the activation-induced cell death process (AICD). (C) Shows prevention of activation of caspase-8 by FLIP binding to the DISC. Apoptosis can also be inhibited on different intracellular levels by other anti-apoptotic proteins (shown in green). (Color version of figure is available online.)

SW480 colorectal adenocarcinoma primary tumor (ATCC order number: CCL-227).

Jurkat-T-cells and SW620 cells were incubated in RPMI 1640 (Biochrom AG, Berlin, Germany), with 10% fetal calf serum (FCS), Biochrom AG, 10 μg/mL streptomycin (Biochrom AG), 100 IU/mL penicillin (Biochrom AG), and 1 mM sodium pyruvate (Biochrom AG).

Coincubation of SW 620 Cells and Jurkat-T-cells

Jurkat-T-cells, 2.5×10^4 were added to SW620 cells, 2.5×10^4, which had been grown in a 24-well plate overnight. They were incubated for 24 h at 37°C/5%CO_2. Next, the medium was taken out of the wells, and centrifuged at 300 *g* for 5 min to collect any cells in suspension. The well was briefly filled with trypsin/EDTA (Biochrom AG, Berlin, Germany) and subsequently washed with PBS (Biochrom AG) to collect remaining cells.

Immunofluorescence

Detection of FAS-ligand expression was shown with FAS-ligand antibody (N-20, SC-834; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and secondary antibody (Alexa Fluor 633; Invitrogen, Paisley, UK). Cell nuclei were stained with DAPI Vector shield (Vectashield DAPI, H-1200; Vector, Burlingame, CA) according to the manufacturer's instructions.

Jurkat-T-Cells Transfection

Three days prior to transfection, the Jurkat-T-cells were kept at a density of 7.5×10^5/mL to ensure they were in the log growth phase. Twelve hours before transfection, the Jurkat-T-cells were placed in 12 mL fresh medium. Immediately before beginning with the transfection, 0.5 mL of a suspension of Jurkat-T-cells (2×10^5/mL in RPMI 1640 without any additives) was placed in the wells of a 24-well plate; 88 μL of rehydrated X-tremeGENE Q2 (Roche, Grenzach, Germany) was diluted with 1012 μL of RPMI 1640 (without additives). Afterwards, 100 μL was separated to prepare a negative control. Twenty μg of the FLIP-plasmid [26] and 20 μg of the pHcRed C1 vector plasmid (Clontech Europe, Saint-Germain-en-Laye, France) were diluted to a total volume of 1000 μL with the DNA dilution buffer supplied with the X-tremeGENE Q2. The diluted DNA solution was incubated for 8 min and then mixed with the transfection reagent whilst the negative control was prepared by adding 100 μL transfection reagent with 100 μL DNA-free DNA dilution buffer. Both were incubated for a further 8 min. After incubation, 100 μL of the mixed contents was added per well, while two wells were used for the previously prepared negative control.

Two wells were incubated with completely untreated Jurkat-T-cells. The cells were incubated for 4 h at 37°C/5%CO_2, then 0.5 mL of RPMI 1640 with 20% FCS was added and the cells incubated for another 24 h. The same was done with 40 μg of pcDNA5-FRT plasmid and 40 μg of pHcRed C1 plasmid.

FACS Analysis

The cell-pellets were resuspended in PBS and incubated for 15 min on ice with the CD3-PC7 kit (Beckman/Coulter Biomedicals, Krefeld, Germany. centrifuged at 300 *g* for 5 min and incubated again for 5 min with the AnnexinV-FITC/7AAD kit. Positive controls for the AnnexinV-FITC/7-AAD kit (Beckman/Coulter Biomedicals, Krefeld, Germany) were made with Jurkat-T-cells, which had been coincubated with activating FAS-antibodies (Anti-Fas clone CH11; Millipore, Schwalbach, Germany).

The FACS (FC500; Beckman Coulter Inc., Brea, CA) was configured with a sample of pure Jurkat-T-cells, and the control samples prepared for the AnnexinV-FITC/7AAD kit and the CD3-PC7 kit. Then the samples were measured in the FACS and analyzed with Beckman Coulter Software "Analysis."

RESULTS

In order to demonstrate the expression of the transmembrane protein, FAS-ligand, in SW620 colorectal adenocarcinoma cells [34], we performed a detection procedure utilizing a primary FAS-Ligand antibody (N-20; Santa Cruz) and an infrared-conjugated secondary antibody (Alexa Fluor 633; Invitrogen) (Fig. 2). Figure 3 shows the negative control for FAS-Ligand expression in SW620 cells treated without primary FAS-ligand antibody.

Tumor Counterattack of SW 620 Cells Against Jurkat-T-Cells *In Vitro*

The objective of the experimental design was to confirm the tumor counterattack of SW620 colorectal adenocarcinoma cells regarding FAS sensitive Jurkat-T-cells *in vitro* [18]. After 24 h coincubation with SW620 cells, 19.89% of Jurkat-T-cell population was in the process of apoptosis, detected with the AnnexinV-FITC/7AAD Kit in FACS analysis (Fig. 4).

FLIP Transfection and Red-Express Co-transfection

To verify the transfection rate of the intracellular protein, FLIP [35], we attached a vector of the red fluorescent reporter protein pHcRed C1, which can be easily detected by FACS-analysis. As described in the literature, cotransfection of a so called Red-express vector is highly significant with cotransfection of other proteins [36]. FLIP and pHcRed C1 cotransfection achieved transfection rates between 40.31% and 59.22% (Figs. 5 and 6).

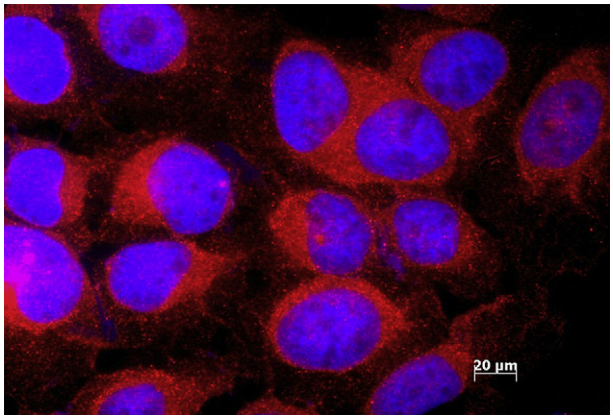


FIG. 2. Detection of FAS-ligand expression on SW620 cells, red colored and SW620 cell nuclei, colored purple. (Color version of figure is available online.)

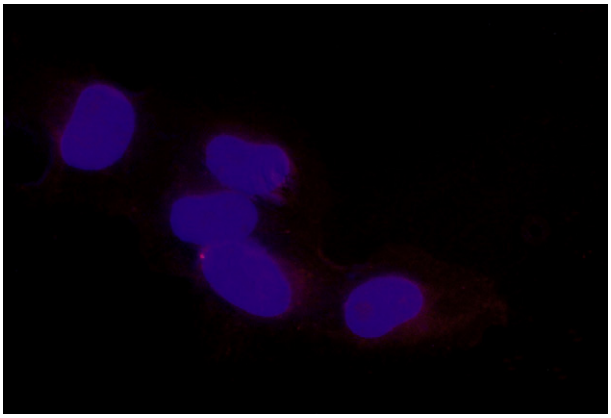


FIG. 3. SW620 FAS-ligand negative control, SW620 cell nuclei, colored purple. (Color version of figure is available online.)

Regain Jurkat-T-Cell Survival After FLIP Transfection in Coincubation with SW620 Cells

To act against the tumor counterattack of SW620 cells *via* the FAS/FAS-ligand pathway, the DISC cascade was inhibited by FLIP transfection of the Jurkat-T-cells.

If FLIP-transfected T-cells are involved and activated in an immunological process, the apoptosis of these cells is prevented by the FLIP-induced blockade of the DISC. Since different malignant tumor types are expressing high levels of Fas-ligand, they can use the highly sensitive FAS receptor of T-cells as a target to eliminate the attacking T-cells *via* the Fas/Fas-ligand pathway. In contrast, FLIP transfected T-cells would be resistant with regard to this target and could thus escape from the tumor counterattack.

FLIP transfection of Jurkat-T-cells leads to a dramatic reduction of apoptosis during coincubation with SW620 cells under identical experimental conditions, demonstrating proof of tumor counterattack (Fig. 4). The AnnexinV-FITC/7AAD Kit detects a fluorescent cell population of Jurkat-T-cells below four percent (Figs. 7 and 8).

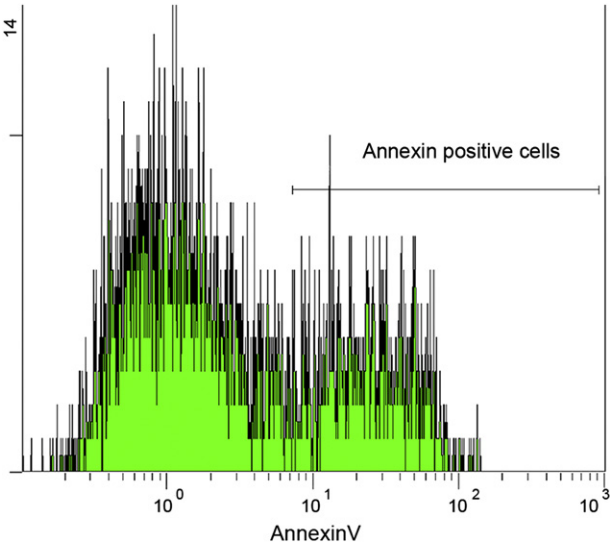
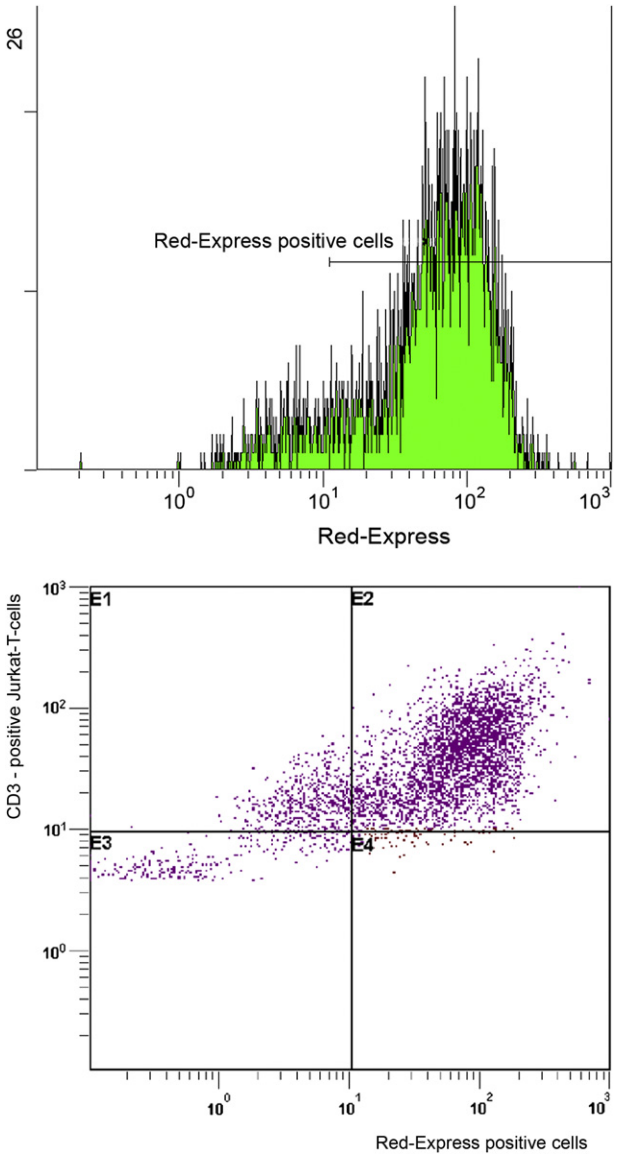


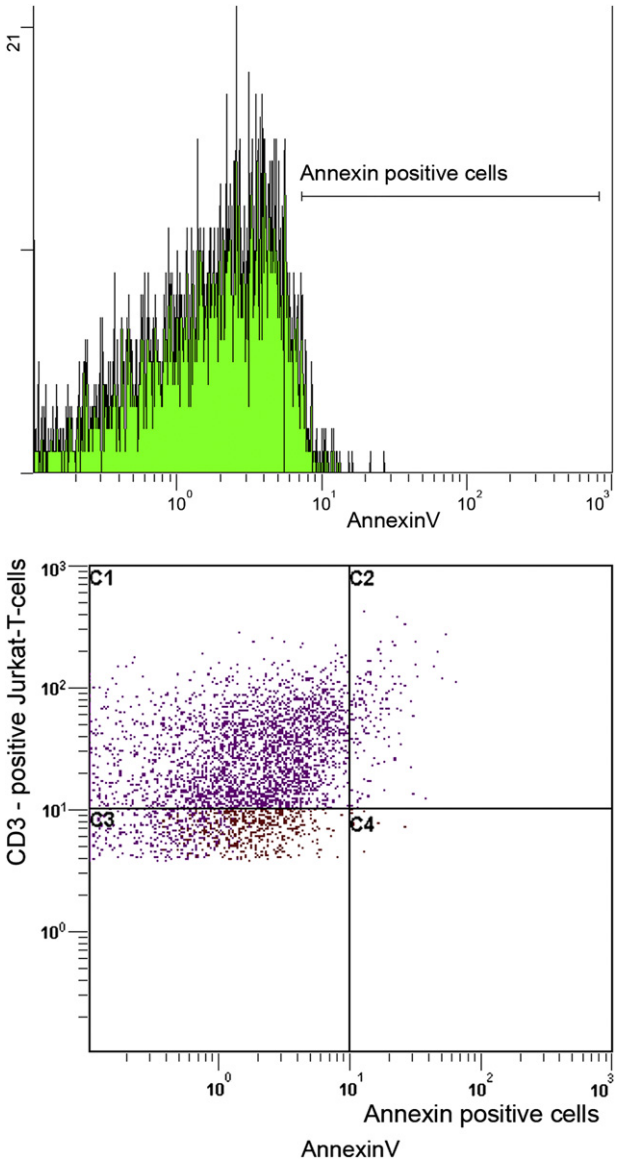
FIG. 4. CD3 positive Jurkat-T-Cells with 19.89% concurrent AnnexinV-FITC/7AAD staining after 24h coincubation with SW620 cells. (Color version of figure is available online.)



FIGS. 5 AND 6. Jurkat-T-cells after cotransfection with FLIP and reporter protein pHcRed C1 (red-expresses vector) with 59.22% transfection rate. (Color version of figure is available online.)

DISCUSSION

In clinical practice, the treatment of malignant tumors is currently dominated by surgical, chemotherapeutic and radiation modalities. Very few successful approaches have been developed involving tumor-specific immune therapies that selectively attack the tumor and are also systemically effective for cases of lymphatic and hematogenic metastasis [37].



FIGS. 7 AND 8. 3.8% Annexin V positive FLIP transfected CD3 positive Jurkat-T-cells after coincubation with SW620 cells. (Color version of figure is available online.)

A trend in basic science research for the therapy of systemic malignant tumors toward the additional modulation of the body's own immune system has been observed [38]. As many malignant tumors are not recognized until they have already achieved a clinically advanced stage, often with metastatic disease, it is our opinion that an improvement in the immune system's ability to deal with tumor cells should be a trend-setting strategy in modern tumor therapy [39–48].

The establishment of a malignant cell population is a function of the critical balance between the local activation and suppression of the immune system. An imbalance toward immune suppression at the point of tumor formation significantly elevates the likelihood of malignant cells surviving [15, 48, 49]. A powerful mechanism for the local induction of immune suppression lies in the ability of many tumors to express FAS-ligand [13–15, 18–23]. The existence of this tumor cell survival strategy, in which the tumor cells induce activated lymphocytes to undergo apoptosis *via* the FAS/FAS-ligand system, is currently controversial [12, 18, 50–55].

Therapeutic options involving the induction of tumor cells to undergo apoptosis *via* so-called death receptors (DR) such as FAS, tumor necrosis factor receptor (TNFR) and TNF-related apoptosis-inducing ligand receptor (TRAIL) has been described in the current literature for various tumor types [56, 57].

A current paper published by Alagkiozidis *et al.* [58] demonstrated in an animal model that chemotherapy with Doxil (liposomal doxorubicin) in combination with IL-18 resulted in a significant up-regulation of FAS receptors and an elevation in the FAS sensitivity of ID8 ovarian cancer cells with a subsequent, significant reduction in tumor growth. The authors determined that the tumor regression was caused by T-cell-lysis *via* the FAS/FAS-ligand system [58].

Another current paper, published by Lahiry *et al.* [59], demonstrated that theaflavin led to the activation of FAS-receptor/caspase-8-mediated apoptosis in p53-mutated human breast cancer cells. As such, current experimental approaches for the therapy of malignant tumors can lead to tumor elimination *via* modulation of the FAS/FAS-ligand system in the tumor through increased expression and sensitivity of the FAS receptor.

The study by Lahiry *et al.* did not examine whether the tumor apoptosis was actually induced by the medication (theaflavin) itself, or whether the medication was able to elevate the FAS-sensitivity and expression, resulting in lysis *via* T-cell-mediated apoptosis [59].

It is certainly possible that the tumor cells' elevated FAS-expression and sensitivity rendered their FAS-ligand-mediated survival strategy irrelevant. No studies exploring this option have been found in the current literature.

A study by Osada *et al.* [60] involved the treatment of T-cells from patients suffering from oxaliplatin-resistant colon carcinoma with the bispecific antibody, MEDI-565, a construct of a bispecific single-chain antibody. It is composed of a humanized anti-CEA single-chain antibody [61] and a human CD3ε-specific single-chain antibody, derived from the mouse monoclonal antibody L2K [62]. The authors carried out a coincubation of the T-cells, pretreated with the MEDI-565 antibody, with tumor cells cultivated from the patient after treatment with oxaliplatin.

They were able to demonstrate significantly elevated sensitivity of the tumor cells to T-cell-mediated tumor

lysis. The authors concluded that the observed tumor regression was caused by an elevation in the cytotoxicity and proliferation of the MEDI-565-treated T-cells. The elevated proliferation and cytotoxicity were explained by an observed up-regulation of CD69 and CD25, which was only seen in the MEDI-565-treated cells and not in the T-cell control group.

Following coincubation of the MEDI-565-treated T-cells with the tumor cells, the authors were also able to demonstrate elevated percentages of granzyme B- and FAS-ligand-expressing CD8+ T-cells.

The novel approach of our study was to demonstrate a resistance of T-cells to tumor-induced apoptosis *via* the FAS/FAS-ligand mechanism by transfecting the T-cells with FLIP. The inclusion of FLIP in the DISC leads to a competitive inhibition of the AICD [23, 26–28, 63]. High levels of FAS-ligand expression has been detected in various types of malignant tumors (colon cancer, breast cancer, ovarian cancer, malignant melanoma, esophageal carcinoma, gastric adenocarcinoma, glioblastoma, basal cell carcinoma, human lung carcinoma, renal carcinoma, pancreatic adenocarcinoma). Furthermore, the so-called tumor counterattack is a survival strategy for tumor cells [13, 14, 18, 22, 64–70]. In consequence, the inhibition of the tumor-mediated apoptosis of tumor-infiltrating lymphocytes *via* the FAS/FAS-ligand system may present an additional approach for the immunological therapy of these malignant diseases.

In other words, the tumor cell-induced apoptosis of FLIP transfected T-cells may be prevented by the FLIP induced blockade of the DISC. This mechanism *via* the Fas/Fas-ligand pathway could prevent the FLIP transfected T-cells from being destroyed by the tumor counterattack.

First, we were able to recreate the tumor counterattack described in the literature in a coculture of Jurkat-T-cells with SW620 colorectal adenocarcinoma cells [12, 18, 29, 50, 71]. Our hypothesis that FLIP-transfection would result in a resistance to FAS-receptor-mediated, tumor-induced apoptosis of Jurkat-T-cells was able to be confirmed in the coculture of Jurkat-T-cells with SW-620 cells.

Our results demonstrate that FLIP-transfection can effect a resistance of Jurkat-T-cells to FAS-induced apoptosis. Therefore, if Jurkat-T-cells may be able to trigger the lysis of tumor cells by some other mechanism aside the FAS/FAS-ligand pathway, such as perforin, granzyme B, TRAIL, or some other as-of-yet unknown cytotoxic mechanism such as suggested by Cullen *et al.* [72], this may enable them to effectively eliminate tumor cells.

Recently published papers have deemed perforin and granzyme B to be important mechanisms for T-cell-mediated elimination of tumor cells [25, 72]. The modulation of the body's own immune system could represent an effective systemic tumor-therapy in the future. Certainly, an uncontrolled blockade of the homeostasis of T-cell populations by FLIP-transfection *in vivo* would act similarly as an autoaggressive disease. Regarding a targeted use of FLIP-transfected T-cells, the decisive step could be the combination of improved tumor-recognition through vaccination, such as through the bispecific antibody MEDI-565 for the recognition of CEA-positive tumors [62] and the generation of a resistance to the tumor-induced apoptosis in T-cells caused by activation of the FAS/FAS-ligand system.

An application in humans seems to be possible through the *ex vivo* modulation of a patient's lymphocytes. The FLIP protein could be stably transduced into the T-cell genome using lentiviral vectors [73], and the transduced T-cells then treated with tumor-specific antibodies before being returned to the patient.

The T-cells' resistance to FAS-mediated apoptosis caused by the FLIP-transduction could be directed by the inclusion of a tetracycline-responsive promoter [74], thereby allowing the longevity of the *ex-vivo* modified T-cells to be controlled. We plan to carry out further studies in a humanized animal model [75].

CONCLUSION

The reduction in the apoptotic rate of Jurkat-T-cells in coincubation with SW620 colorectal adenocarcinoma cells following transfection with FLIP demonstrates that the blockade of the tumor-mediated apoptosis of T-cells can effectively combat the so-called tumor counterattack, which is an important tumor survival strategy. It is our opinion that the combination of immune-modulating treatment approaches may represent the future of tumor therapy. The antitumoral regeneration of the patient's immune system will be at the forefront of this research.

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