GM1 and Tumor Necrosis Factor-α, Overexpressed in Renal Cell Carcinoma, Synergize to Induce T-Cell Apoptosis

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Abstract

The ability to induce T-cell apoptosis is one mechanism by which tumors evade the immune system, although the molecules involved remain controversial. We found that renal cell carcinoma (RCC)–induced T-cell apoptosis was inhibited by >50% when cocultures were performed with ganglioside-depleted tumor cells, caspase-8–negative lymphocytes, or anti–tumor necrosis factor-α (TNFα) antibodies, suggesting that tumor gangliosides synergize with signals delivered through TNFα death receptors to mediate T-cell killing. The synergy between tumor-derived TNFα and the RCC-overexpressed ganglioside GM1 for killing resting T cells is corroborated by studies using purified GM1 and rTNFα, which indicate that a 48-hour pretreatment with the ganglioside optimally sensitizes the lymphocytes to a TNFα-induced apoptotic death. However, activated T cells, which synthesize TNFα themselves, can be killed by exogenous GM1 alone. RelA-overexpressing lymphocytes are protected from GM1 plus TNFα-mediated apoptosis, a finding consistent with our previous studies indicating that gangliosides inhibit nuclear factor-κB activation. These results are clinically relevant because, similar to T-cells cocultured with GM1-overexpressing RCC lines, T cells isolated from the peripheral blood of patients with metastatic RCC are also heavily coated with that tumor-shed ganglioside. This population of patient cells, unlike T cells isolated from normal donors, is highly susceptible to apoptosis induced by rTNFα or by metastatic patient sera, which contain elevated levels of the cytokine. This report thus extends our previous studies by demonstrating that tumor-derived TNFα enhances RCC apoptogenicity not only by inducing ganglioside synthesis but also by initiating receptor-dependent apoptosis in T cells in which the nuclear factor-κB activation pathway has been inhibited by GM1. [Cancer Res 2008;68(6):2014–23]

Introduction

Numerous studies indicate that tumors do elicit immune responses, with tumor-specific T-lymphocytes clonally expanding in many individuals harboring malignancies (1, 2). These initial responses seem to be either short lived or ineffective, however, because the majority of tumors progress and metastasize, and most patients eventually die of their disease (3). Our laboratory studies tumor-induced T-cell apoptosis as a mechanism by which renal cell carcinoma eludes immune destruction: when examined by *in situ* terminal nucleotidyl transferase (TdT)–mediated nick end labeling (TUNEL) analysis, 30% to 100% of renal cell carcinoma (RCC) tumor-infiltrating lymphocytes (TIL) are Annexin V/amino actinomycin D–positive (4). The systemic reach of tumor-mediated immunosuppression is suggested by the fact that even patient peripheral blood T cells are susceptible to activation-induced cell death upon explantation (4). It is the tumors themselves that mediate these effects because T lymphocytes undergo the same physiologic changes associated with apoptosis following *in vitro* culture with cancer cell lines (5–7).

A variety of tumors have been reported to express aberrantly elevated levels of FasL, TNF-related apoptosis-inducing ligand, or CD70, ligands that can mediate proapoptotic effects upon binding their specific cognate receptors (8–10). Our laboratory has also recently elucidated a role for soluble, tumor-associated products in mediating T-cell apoptosis (11, 12). Further studies have shown that shed, overexpressed tumor-associated gangliosides play a significant part in these events (13–16). Renal cell carcinomas, for example, display increased levels of GM1, GM2, and GD1a (17). Although there is precedence for gangliosides suppressing antitumor immunity (18, 19), the mechanism by which they act is unclear.

Our previous studies indicated that gangliosides purified from RCC supernatants depressed nuclear factor-κB (NFκB) activation in lymphocytes, and also triggered cytochrome *c* release and induced apoptosis if coincubated with T cells for extended periods (7, 11, 12, 20). All of these effects were also mediated by the tumor cells themselves, but were abrogated when tumor ganglioside synthesis was inhibited prior to coculture by pretreatment with the glycosylceramide synthase inhibitor 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol. Our finding that caspase-8–negative Jurkat cells were also largely resistant to a ganglioside-overproducing RCC line suggested that another tumor-derived product, likely acting through a death receptor, might also synergize with glycosphingolipids to mediate T-cell death. Interestingly, loss of function mutations in the von Hippel-Lindau (VHL) tumor suppressor protein are common in clear cell RCC (21, 22). Because tumor necrosis factor-α (TNFα) is up-regulated in tumors harboring the mutation (23), most RCCs synthesize TNFα constitutively. Reports demonstrating that TNFα stimulates ganglioside expression in several normal cell types (24) led to our experiments revealing that TNFα could augment tumor killing of cocultured T-lymphocytes via a mechanism involving increased tumor ganglioside synthesis (13). Left unresolved, however, was the

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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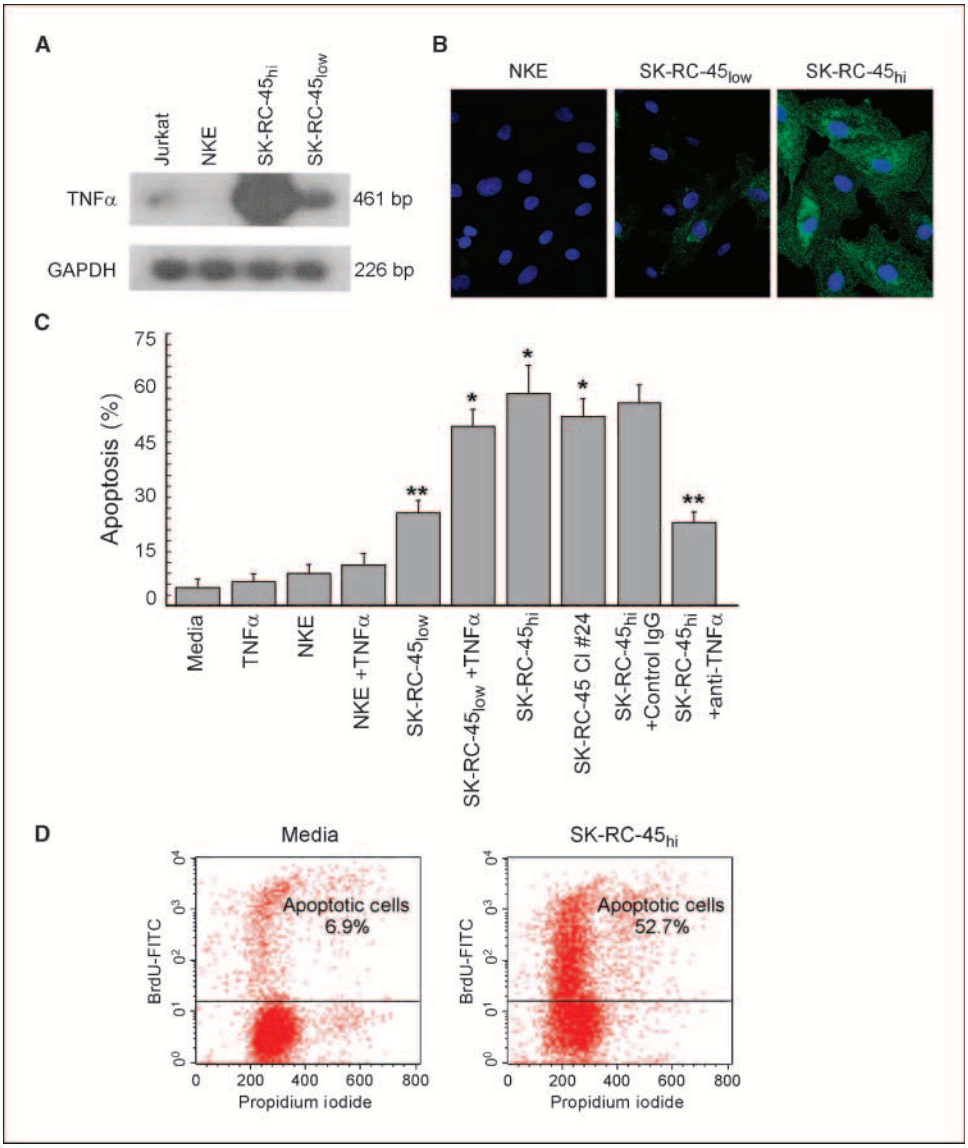

Figure 1. RCC tumor cells expressing elevated levels of TNFα exhibit enhanced apoptogenicity for T cells. *A,* RNA preparations from NKE cells and two RCC clones were subjected to RT-PCR using 5′ and 3′ primers for TNFα, demonstrating the isolation of an SK-RC-45 variant clone (*SK-RC-45$_{hi}$*) expressing ~7-fold higher levels of the cytokine than SK-RC-45$_{low}$. Primers for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as a control for RNA levels in each sample. *B,* NKE, SK-RC-45$_{low}$, and SK-RC-45$_{hi}$ cells were subjected to immunostaining with 2 μg/mL of anti-TNFα, followed by Alexa 488–labeled secondary antibody. Nuclei were revealed by DAPI staining. *C,* resting T cells were cocultured for 72 h with NKE cells, SK-RC-45$_{hi}$, SK-RC-45$_{low}$, TNFα-transfected SK-RC-45$_{low}$ (clone 24), or SK-RC-45$_{hi}$ in the continuous presence of anti-TNFα antibodies (10 μg/mL), at which time, lymphocytes were evaluated by TUNEL for apoptosis. *Columns,* mean of three experiments; *bars,* SE (*, $P < 0.001$; **, $P < 0.01$ versus medium alone). *D,* resting T cells incubated in medium or cocultured with SK-RC-45$_{hi}$ for 72 h were analyzed for tumor-induced apoptosis by TUNEL analysis. Typical data obtained by flow cytometry which was used to generate the results in *C*, and all subsequent experiments assessing T cell apoptosis under various experimental conditions.

molecular pathway(s) by which these TNFα- and ganglioside-producing tumor cells mediated their toxic effects. Here, we report that RCC-derived products GM1 and TNFα, in both their tumor-associated and purified forms, could induce T-cell dysfunction by synergistically activating the receptor-dependent apoptotic pathway in lymphocytes. The physiologic relevance of these studies stems from our findings that, unlike lymphocytes from healthy donors, peripheral blood T cells isolated from patients with RCC stain strongly for GM1, and are induced to apoptosis when treated *in vitro* with either TNFα or with RCC patient serum, which we find contains elevated levels of TNFα.

Materials and Methods

Reagents. Anti-TNFα, anti-TNFR1, anti–caspase-8, anti–caspase-9, and anti-FADD antibodies were rabbit polyclonal antibodies from Santa Cruz Biotechnology, and murine monoclonal antiactin was purchased from Abcam. Horseradish peroxidase–conjugated sheep anti-mouse and donkey anti-rabbit immunoglobulin antibodies were from Amersham. FITC-labeled cholera toxin was from List Biologicals, and anticholera toxin antibody was from Calbiochem. Protein Sepharose beads were from Pierce. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were from Sigma-Aldrich. Monoclonal anti-CD3 (OKT3) and monoclonal anti-CD28 (BD Immunocytometry Systems) were from Ortho Biotech. Caspase-8 inhibitor I (IETD-CHO), used at 50 μmol/L, was from Calbiochem. GM1 was from Matreya. Gangliosides were isolated from RCC tissues, purified and subjected to high-performance liquid chromatography (HPLC) as previously described (25). Human recombinant interleukin-2 [Aldesleukin (Proleukin), CHIRON Corporation] was used at 20 units/mL to maintain the viability of activated T cells.

Plasmids and transfection. TNFα was cloned from activated T cells, and the cDNA was inserted into the *Hin*dIII and *Xba* sites of PcDNA3. Jurkat cells were transfected and selected with G418 as described previously (20) and clones generated by limiting dilution were characterized for cytokine production using a TNFα ELISA kit (R&D Systems). The RelA cDNA and RelA-transfected Jurkat cells were prepared as described previously (20).

Cell culture. Peripheral blood was obtained and T cells purified and activated as described previously (13). The Jurkat leukemic T-cell line was purchased from American Type Culture Collection. Caspase-8–negative Jurkat cells were a gift from John Blenis (Department of Cell Biology, Harvard Medical School, Boston, MA; ref. 26). The long-term renal cell carcinoma line (SK-RC-45; ref. 27) was obtained, grown, and used experimentally in cocultures as described previously (13). A variant clone of SK-RC-45 with enhanced apoptogenicity for resting T cells was isolated from the parental stock, expressed elevated levels of TNFα, and hence, was designated SK-RC-45_{hi}. SK-RCC-45 clone 24 was derived by transfecting the parental SK-RC-45_{low} cells with a TNFα-encoding plasmid. A normal kidney epithelial (NKE) cell line was used as a negative control in coculture experiments.

Immunofluorescence. Tumor lines were immunostained to assess GM1- and TNFα expression levels. Cells were fixed in paraformaldehyde and permeabilized with Triton X-100 (Sigma). Cells were blocked with 2% fetal bovine serum/1% bovine serum albumin, and incubated overnight at 4°C with either FITC-labeled cholera toxin or anti–TNFα antibodies. Cells were subsequently incubated with the appropriate, labeled secondary antibodies (Molecular Probes), washed, and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei.

Analysis of DNA fragmentation by TUNEL analysis. Cells were fixed in 1% paraformaldehyde, and were stained and analyzed for apoptosis using the APO-BRDU system (Invitrogen), as described previously (20).

Cell lysates and analysis of protein by Western blotting. Cell pellets were resuspended in lysis buffer and subjected to Western analysis as described (7).

Analysis of TNFα expression using reverse transcription-PCR and chemiluminescent ELISAs. Total cellular RNA was extracted and subjected to semiquantitative reverse transcription-PCR (RT-PCR) analysis according to previously described methods and cycling conditions (28). The primer and probe sequences for TNFα and glyceraldehyde-3-phosphate dehydrogenase were as described (28, 29). Sera isolated from 19 patients with RCC and 6 normal controls were assessed for their TNFα concentrations using the QuantiGlo Human TNFα chemiluminescent ELISA kit (R&D Systems) according to the manufacturer's instructions.

HPLC analysis of tumor gangliosides. Tumor gangliosides isolated from SK-RC-45_{low} and SK-RC-45_{hi} were subjected to HPLC analysis as previously described (13).

Statistical analysis. Student's *t* test (two paired samples for mean or two samples using equal variances) was used to determine *P* using Microsoft Excel Software (version 2003). SE was calculated from SD using Microsoft Excel software.

Results

RCC tumor cells kill resting T-lymphocytes by a mechanism dependent on gangliosides and the TNFα receptor/ligand pair. Gangliosides are overexpressed by a variety of tumor types (19), and have been reported to mediate cytotoxic effects by disrupting the mitochondrial function of target cells (7, 30). The data presented in Fig. 1 support the notion that tumor gangliosides also contribute to T-cell apoptosis by a mechanism exhibiting death receptor dependency. When NKE cells and two RCC cell lines differing vastly in their TNFα expression levels (SK-RC-45_{low} and SK-RC-45_{hi}; Fig. 1*A* and *B*) were compared for their relative abilities to kill cocultured resting T cells *in vitro*, the NKE cells were found to have a negligible effect whereas the tumor lines mediated apoptosis in proportion to their levels of TNFα expression (Fig. 1*C*). Unlike the NKE cells, which even if pretreated with TNFα did not overexpress gangliosides and could not induce T-cell apoptosis, SK-RC-45_{hi} (13) and the TNFα-transfected SK-RC-45 clone 24 stimulated an average of 55% to 60% of the lymphocytes to TUNEL positivity (Fig. 1*C* and *D*). This was more than twice the level of T-cell apoptosis induced by the parental SK-RC-45_{low} line, which also

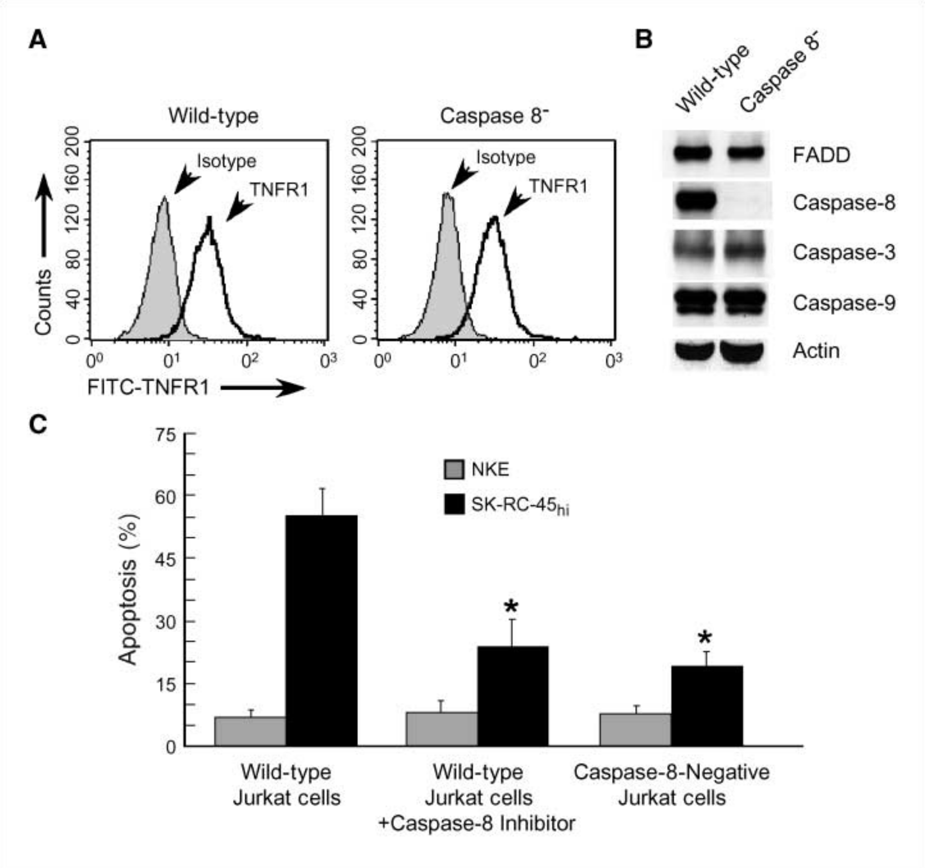

Figure 2. Caspase-8 negativity protects T cells from apoptosis induced by SK-RC-45_{hi}. *A,* wild-type and caspase-8–negative Jurkat cells were stained with anti-TNFR1 to assess TNFR1 receptor expression by flow cytometric analysis, as described in Materials and Methods. *B,* cytoplasmic lysates made from wild-type and caspase-8–negative Jurkat cells were subjected to Western blot analysis using 10 μg protein/lane and antibodies to FADD, caspase-8, caspase-9, caspase-3, and actin. *C,* wild-type Jurkat cells, caspase-8–negative Jurkat cells, or wild-type Jurkat cells pretreated for 90 min with 50 μmol/L of caspase-8 inhibitor I were incubated with SK-RC-45_{hi} for 72 h prior to analyzing the lymphocytes by TUNEL for tumor-induced apoptosis, as described in Materials and Methods. *Columns,* mean of three experiments; *bars,* SE (*, $P < 0.001$).

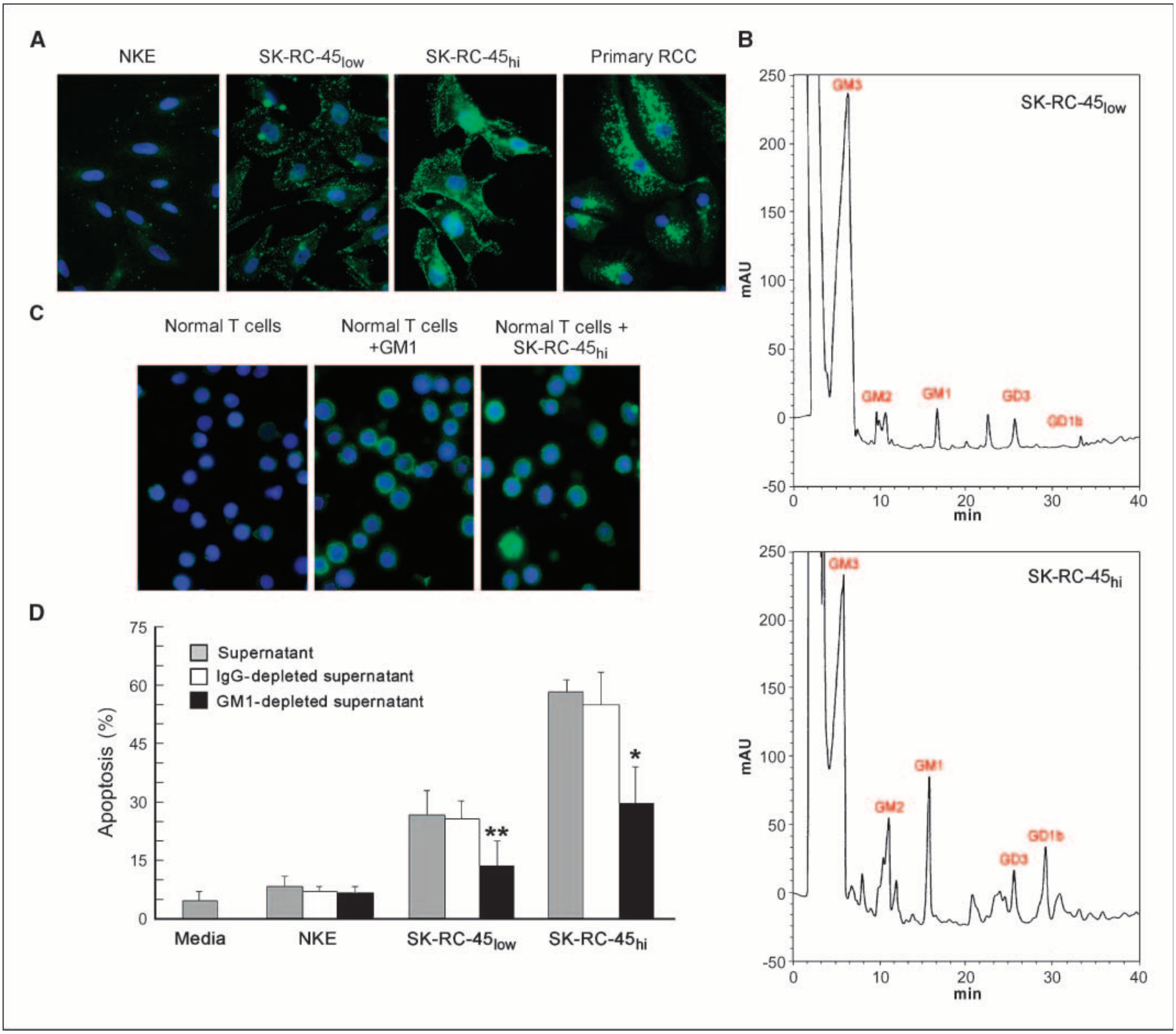

Figure 3. TNFα-producing RCC cells overexpress the ganglioside GM1, which adheres to T-cell membranes and participates in lymphocyte killing. *A,* NKE cells, SK-RC-45$_{low}$, SK-RC-45$_{hi}$, and primary RCC tumor cells were stained with 2 μg/mL of FITC-labeled cholera toxin and examined by fluorescent microscopy for GM1 expression, as described in Materials and Methods. *B,* HPLC profiles of gangliosides isolated from SK-RC-45$_{low}$ and SK-RC-45$_{hi}$ cell lines were obtained as described in Materials and Methods, demonstrating the elevated levels of GM1 in SK-RC-45$_{hi}$. *C,* normal, peripheral blood resting T-cells were either cocultured with SK-RC-45$_{hi}$, or incubated in the presence or absence of 25 μg/mL of GM1 for 2 h, prior to staining the lymphocytes with FITC-labeled cholera toxin to assess levels of T-cell membrane–associated GM1. *D,* conditioned medium from confluent cultures of NKE cells, SK-RC-45$_{low}$, or SK-RC-45$_{hi}$ were immunodepleted or not of GM1 with cholera toxin/anti–cholera toxin/protein A-Sepharose beads. Resting T cells from normal donors were then cultured for 72 h in each (diluted 50% with fresh medium), and then subjected to TUNEL analysis to assess the involvement of GM1 in apoptosis induced by tumor supernatants. Supernatants treated with control IgG and protein A-Sepharose beads served as negative controls. *Columns,* mean of three experiments; *bars,* SE (*, $P < 0.005$ vs. GM1-depleted NKE supernatants; **, $P < 0.05$ vs. NKE-depleted supernatants).

became more apoptogenic if pretreated with TNFα prior to a coculture experiment (Fig. 1*C*).

In our earlier published reports, the role of TNFα in mediating the apoptosis of resting T cells was examined only in the context of the cytokines' capacity to stimulate tumor ganglioside synthesis. However, because in those studies, the TNFα used for pretreating the SK-RC-45 was not removed from the tumor cell monolayers prior to initiating the coincubation experiments, the possibility that TNFα might be mediating an additional, requisite proapoptotic activity was not addressed. Here, our determination that anti-TNFα antibodies inhibited RCC-mediated killing of T cells even after TNFα-induced ganglioside synthesis was maximal (ref. 13; Fig. 1*C*), suggested that TNFα was likely playing an additional obligatory role in tumor-induced apoptosis of the lymphocytes. Because numerous laboratories have implicated death ligands expressed by histologically diverse tumors in the apoptosis of TILs (8–10), we thus considered the possibility that TNFα might be mediating this additional proapoptotic effect through the TNFα death receptor.

Caspase-8–deficient Jurkat cells are resistant to the apoptogenic SK-RC-45$_{hi}$ line. Because caspase-8 is a requisite for death receptor–dependent activation of the caspase cascade (31), the use of caspase-8–negative Jurkat cells provided an opportunity to assess the role of TNFR1 in SK-RC-45_{hi}–mediated killing (Fig. 2*A* and *B*). Wild-type and caspase-8–negative Jurkat cells were thus analyzed for DNA breaks by TUNEL following a 72-hour coincubation with adherent SK-RC-45_{hi} monolayers. Wild-type Jurkat cells were highly sensitive to SK-RC-45_{hi}, with an average of 55% of the cocultured lymphocytes testing positive for apoptosis (Fig. 2*C*), although only minimal TUNEL positivity was evident when, as controls, the lymphocytes were exposed to NKE cells (~7.0% apoptosis) or fresh medium alone (3%; data not shown). The defect in T-cell death receptor signaling conferred by the caspase-8 mutation, however, dramatically decreased the susceptibility of that Jurkat cell clone to SK-RC-45_{hi} by ~55%, suggesting that the tumor-derived TNFα, beyond inducing ganglioside synthesis, is additionally acting in a death receptor and caspase-8–dependent manner (Fig. 2*C*). Supporting this conclusion was the even greater inhibition of tumor-induced apoptosis observed (65% inhibition) when SK-RC-45_{hi} cells were coincubated with wild-type Jurkat cells in the presence of caspase-8 inhibitor I (IETD-CHO; Fig. 2*C*). The fact that SK-RC-45_{hi}–induced Jurkat cell apoptosis can be reduced by >50% either by abrogating tumor cell ganglioside synthesis or by inhibiting death receptor–mediated activation of the caspase cascade, indicates that the RCC tumor line synthesizes multiple proapoptotic products (i.e., TNFα and gangliosides) that synergize to induce the apoptosis of Jurkat cells and normal T cells.

RCC tumor cells overexpress GM1 and induce apoptosis of resting T-lymphocytes in proportion to their level of TNFα synthesis. When SK-RC-45_{low} and SK-RC-45_{hi} were compared with a NKE cell line for GM1 expression by immunostaining with FITC-labeled cholera toxin, it was evident that the RCC tumor cells produced much greater amounts of that ganglioside (Fig. 3*A*), and did so in proportion to their TNFα expression levels (13): SK-RC-45_{hi} cells stained much brighter than SK-RC-45_{low}. These finding were confirmed when gangliosides isolated from the two RCC clones were compared for GM1 content by HPLC (Fig. 3*B*). The relevance of the SK-RC-45_{hi} model for studying GM1 participation in RCC-mediated T-cell killing was supported by studies that examined GM1 overexpression by primary RCC. When primary RCC explants from eight different patients were digested into single cell suspensions and analyzed with FITC-labeled cholera toxin for GM1 levels, all were determined to express highly abundant quantities of the ganglioside (representative fluorescent micrograph; Fig. 3*A*). The potential physiologic implications of this overexpression are shown in Fig. 3*C*. Not only do T-cell membranes incorporate GM1 when the purified ganglioside is added to the culture medium (Fig. 3*C, middle plate*), but T cells also become coated with the molecule when they are cocultured with a GM1-overexpressing RCC line (Fig. 3*C, right plate*). The deleterious consequences of T cell contact with exogenous GM1 could be discerned from the experiment depicted in Fig. 3*D*. When normal resting, peripheral blood T-cells were assessed for apoptosis

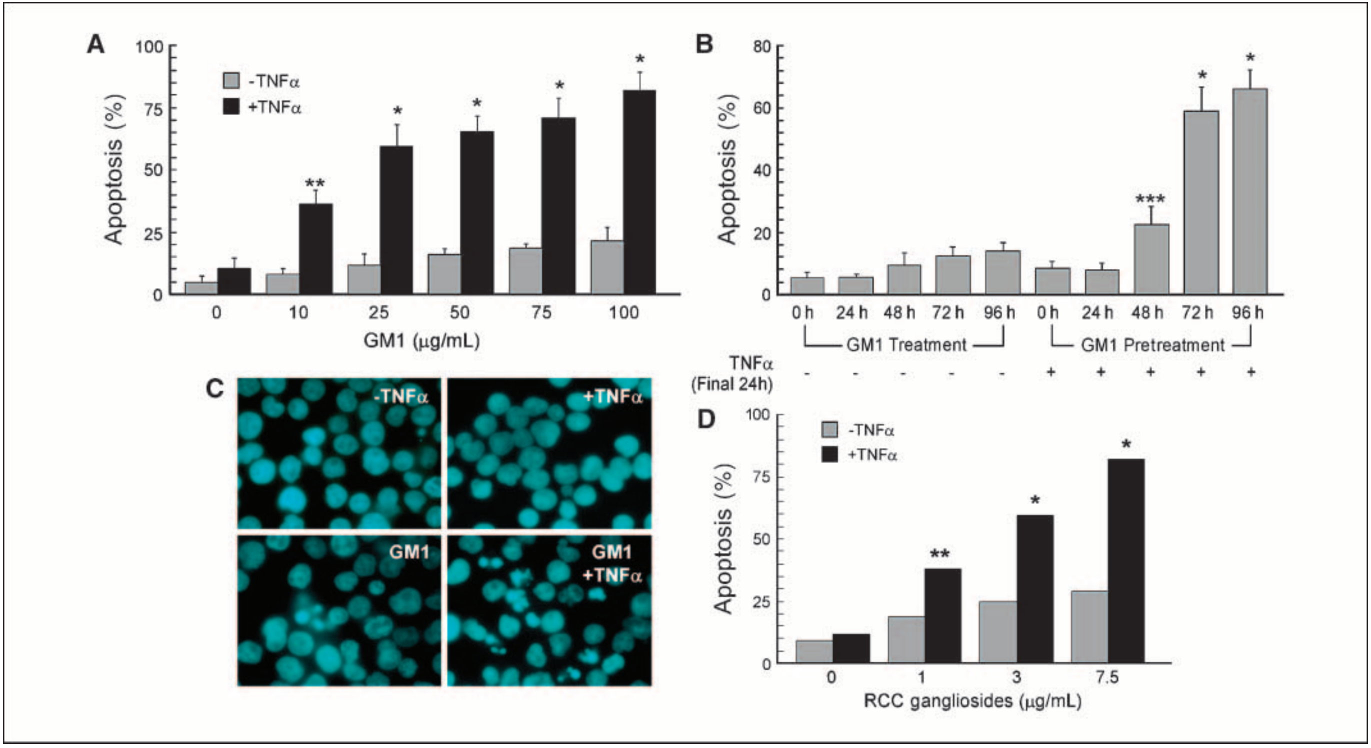

Figure 4. Gangliosides and TNFα synergistically induce T-cell apoptosis. *A,* resting peripheral blood T-cells were treated with escalating doses of GM1 for 48 h prior to stimulating them or not for 24 h with 100 ng/mL of TNFα. Cells were subsequently analyzed for apoptosis by TUNEL. *Columns,* mean of three experiments; *bars,* SE (*, $P < 0.001$; **, $P < 0.01$ vs. 0 μg/mL GM1). *B,* resting peripheral blood T-cells were treated with 25 μg/mL of GM1 for 24, 48, 72, and 96 h, the last 24 h in the presence or absence of 100 ng/mL of TNFα. Cells were subsequently analyzed for apoptosis by TUNEL. *Columns,* mean of three experiments; *bars,* SE (*, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$ vs. 0 h GM1 pretreatment). *C,* resting peripheral blood T-cells were treated or not with 25 μg/mL of GM1 with or without TNFα as described in *A*, prior to being stained with DAPI to quantify apoptotic nuclei by fluorescent microscopy. *D,* RCC gangliosides were isolated from SK-RC-45_{hi} as described in Materials and Methods, and were used at different concentrations to treat resting T cells prior to a subsequent stimulation with 100 ng/mL of TNFα. After 72 h, cells were subjected to TUNEL analysis to quantify apoptosis induced by the different treatments (*, $P < 0.001$; **, $P < 0.01$ vs. 0 μg/mL RCC gangliosides).

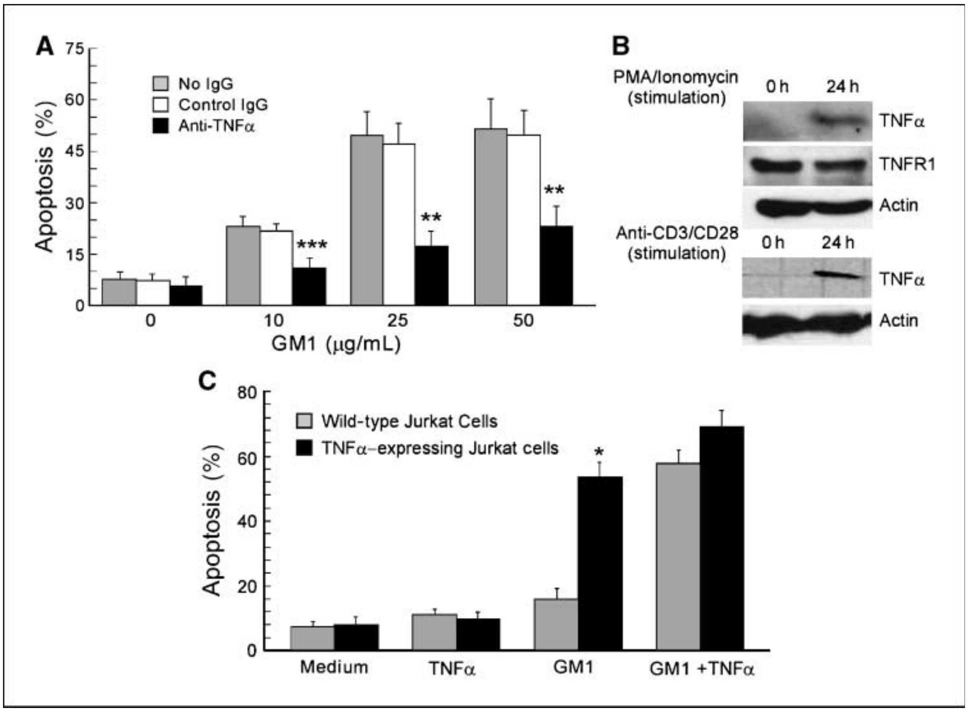

Figure 5. GM1 alone can induce the apoptosis of activated T-cells and TNFα-transfected Jurkat cells. *A,* peripheral blood T-cells preactivated with anti-CD3/anti-CD28 were treated with increasing doses of GM1 (10, 25, 50 μg/mL), in the continuous presence or absence of control IgG or anti-TNFα antibodies for 72 h, prior to being assessed for apoptosis by TUNEL. *Columns,* mean of three experiments; *bars,* SE (**, $P < 0.01$; ***, $P < 0.05$ vs. No IgG). *B,* resting peripheral blood T-cells were stimulated or not with either PMA/ionomycin or anti-CD3/anti-CD28 prior to subjecting whole cell lysates to Western blot analysis using antibodies to TNFα and TNFR1. Actin served as a loading control. *C,* wild-type Jurkat cells and a Jurkat cell line permanently transfected with TNFα-encoding cDNA were treated for 48 h with 100 ng/mL of TNFα alone, 25 μg of GM1 alone, or GM1 for 24 h followed by TNFα for 24 h, at which point, cells were analyzed by TUNEL for apoptosis. *Columns,* mean of three experiments; *bars,* SE (*, $P < 0.001$ vs. medium alone).

following a 72-hour exposure to NKE, SK-RC-45_{low}, or SK-RC-45_{hi} culture supernatants, the conditioned medium from the NKE line was found to have no injurious effect on T-cell viability, whereas the two tumor cell supernatants induced T-cell killing in direct proportion to their individual levels of GM1 synthesis (25% and 55% apoptosis, respectively; Fig. 3*D* and *A*). The specific involvement of GM1 in this killing was clearly demonstrated in Fig. 3*D*, which shows that depletion of GM1 from the tumor cell–conditioned medium by sequential treatment with cholera toxin, anticholera toxin antibody, and protein A-Sepharose beads reduced the apoptogenicity of the tumor supernatants by ~50%, although control IgG and beads had no effect.

Both purified GM1 and purified RCC gangliosides synergize with recombinant TNFα to induce apoptosis of resting T cells. To further evaluate the notion that GM1 and TNFα synergize to induce T-cell apoptosis, resting peripheral blood T cells were incubated for 48 hours with escalating doses of purified GM1, prior to treating them or not for a final 24 hours with 100 ng/mL of TNFα. Only minimal killing was observed when T cells were cultured with either TNFα alone, or with 10 or 25 μg/mL of GM1 alone, although some modest levels of apoptosis were achieved when cells were incubated with higher concentrations of the ganglioside (Fig. 4*A*). Synergy between GM1 and TNFα, however, was observed throughout the range of GM1 provided. The effect became optimal at a GM1 concentration of 25 μg/mL, at which dose GM1 plus TNFα killed 60% of the cocultured T-cells, five times more cells than could be killed by either the ganglioside or TNFα alone (Fig. 4*A*). A time course study was also performed to determine the optimal GM1 preexposure time requisite for maximal synergy (Fig. 4*B*). This experiment revealed that when resting T cells were incubated with 25 μg/mL of GM1 for different time periods prior to adding TNFα for a final 24 hours of culture, a 48-hour preincubation with GM1 (i.e., the 72-hour final time point) was found to provide the maximum apoptotic effect. DAPI staining (32) of resting T cells following exposure to GM1 plus TNFα revealed a significant increase in the percentage of apoptotic nuclei per visual field compared with untreated control cells, or to cells treated with either GM1 or TNFα alone (Fig. 4*C*). Together, these results support the concept that GM1 and TNFα synergistically mediate the apoptotic death of T cells.

We next wanted to determine whether purified RCC-derived gangliosides also synergize with TNFα to mediate T-cell killing. Resting T cells were incubated for 48 hours with increasing doses of a ganglioside preparation purified from the RCC tumor cell line SK-RC-45_{hi}, following which TNFα was added for an additional 24 hours. RCC-derived gangliosides were more potent mediators of T-cell apoptosis than purified GM1, as alone, 7.5 μg/mL of the tumor-derived gangliosides killed 20% of the coincubated T-cells (above background; Fig. 4*D*). However, synergy between RCC gangliosides and TNFα was still very much evident, and was maximal at 7.5 μg/mL of RCC gangliosides. At this concentration, tumor gangliosides synergized with TNFα to kill >80% of the treated T cells (Fig. 4*D*).

GM1 can synergize with T cell–derived TNFα to kill activated T lymphocytes directly. Although 25 μg/mL of GM1 alone was not significantly apoptogenic for resting T cells (Fig. 4*A*), it could induce the apoptotic death of TNFα-expressing activated T-cells (Fig. 5*A*). Although both resting and activated T cells express TNFR1 (Fig. 5*B*), only the activated cells express detectable TNFα (Fig. 5*B*). Thus, it may be that because activated T cells can provide their own source of synergizing TNFα, they can be killed by GM1 alone (Fig. 5*A*). This contrasts with TNFα-deficient, resting T-cells, which can only be killed by GM1 plus TNFα (Fig. 4*A–C*) or by an RCC tumor line that expresses both gangliosides and TNFα (Figs. 1*C, D*, and 3*D*). Indeed, incubation of activated T cells with GM1 in the presence of antibodies to TNFα significantly abrogated the ganglioside-mediated killing (Fig. 5*A*), whereas control IgG had essentially no effect. This line of reasoning was further supported by experiments showing that Jurkat cells treated with GM1 or TNFα did not undergo apoptosis unless the two reagents were used in synergy, in which case, >55% of the cells succumbed (Fig. 5*C*). Jurkat cells engineered to secrete their own source of TNFα, on the other hand, remained viable when cultured in medium or if treated with additional TNFα, but could be induced to apoptosis if treated with 25 μg/mL of purified GM1 alone, the latter likely synergizing with cellular sources of TNFα to mediate the effect (Fig. 5*C*).

Jurkat cells genetically defective for NFκB activation are susceptible to TNFα alone, with GM1 providing no additional effect; conversely, Jurkat cells engineered to overexpress RelA are resistant to the synergistic, apoptotic effects of GM1 and TNFα. Previous studies from our laboratory indicated that RCC tumor gangliosides inhibit PMA/ionomycin–induced NFκB activation (11). To investigate the possibility that inhibition of NFκB might be the mechanism by which GM1 synergizes with TNFα to induce T-cell apoptosis, wild-type Jurkat cells and Jurkat cells transfected with a plasmid encoding the IκBα super-repressor (which inhibits NFκB activation) were compared for their susceptibilities to TNFα, GM1, or both agents in synergy. Neither the wild-type nor the NFκB-defective Jurkat populations were killed by a 72-hour incubation with GM1, and as expected, as many as 51% of the IκBα mutant transfectants died in response to TNFα alone (Supplementary Fig. S1A). Interestingly, unlike the case for wild-type cells, pretreatment with GM1 did not further enhance the ability of TNFα to kill the NFκB-inhibited, IκBα mutant–expressing cells (Supplementary Fig. S1A).

To further assess the notion that GM1 might act by inhibiting NFκB, we tested the possibility that NFκB-overexpressing cells might be resistant to GM1 plus TNFα–mediated apoptosis. RelA-transfected and wild-type Jurkat cells were treated or not with GM1, TNFα, or both in synergistic combination. As compared with GM1 or TNFα, which independently exerted minimal apoptogenicity for either cell line, in synergy, those reagents stimulated ~50% of the wild-type Jurkat cells to undergo apoptosis (Supplementary Fig. S1B). RelA overexpression bestowed significant protection against GM1 plus TNFα, however, as only 20% of the transfectants were killed by the treatment.

Circulating RCC patient T-cells are coated with tumor-derived GM1, and can be induced to apoptosis *in vitro* with patient sera but not with normal sera. Unlike normal T cells which stain only weakly for GM1, but strongly after coincubation

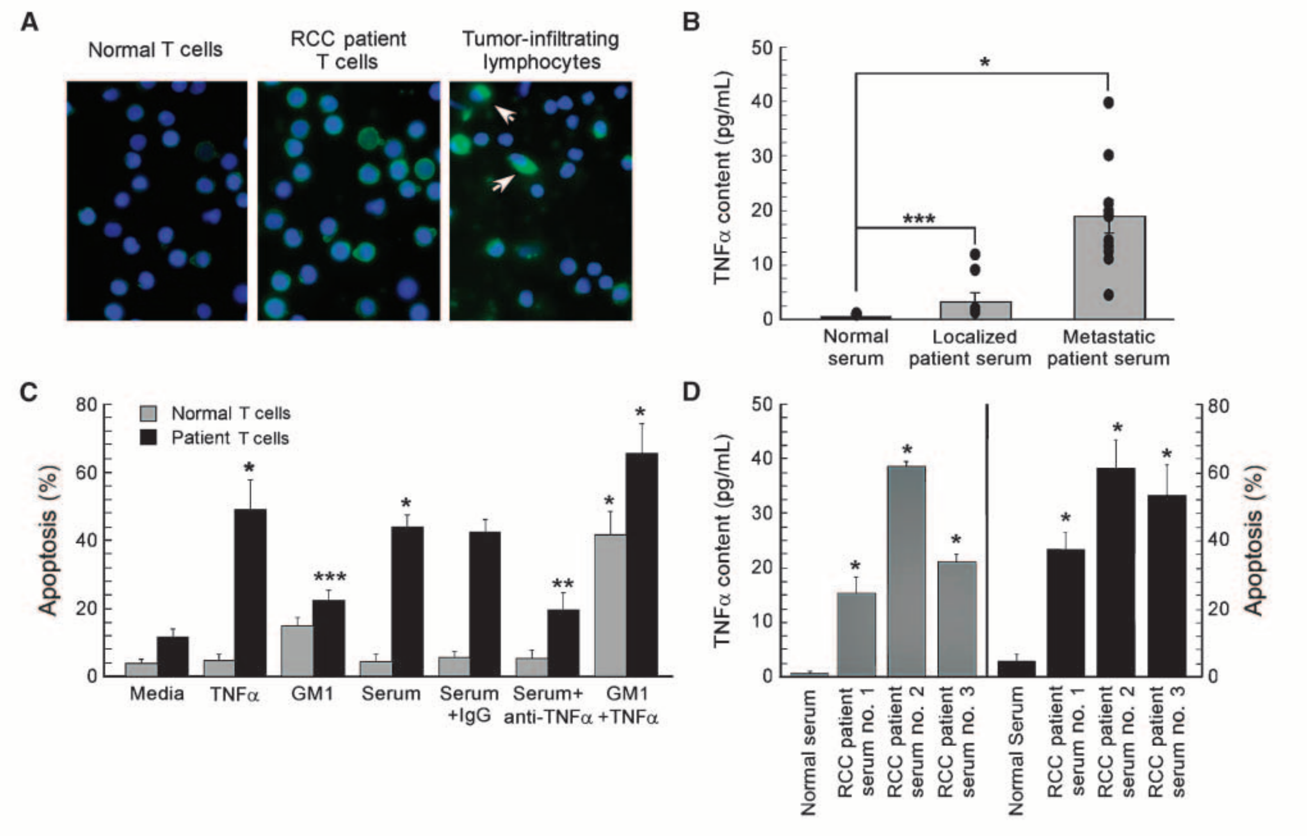

Figure 6. TILs and peripheral blood T-cells isolated from RCC patients were coated with GM1, and could be induced to apoptosis *in vitro* with either TNFα or RCC patient serum. *A,* peripheral blood T-cells isolated from healthy donors and patients with RCC, as well as TILs, were stained with FITC-labeled cholera toxin to assess GM1 levels. *B,* a TNFα chemiluminescent ELISA kit was used to measure the TNFα levels present in the sera obtained from 6 healthy donors, 8 RCC patients with localized disease, and 11 RCC patients with metastatic disease. *Columns,* mean of 6 to 11 samples; *bars,* SE; *dots,* TNFα levels present in the serum of a single individual (*, $P < 0.001$; ***, $P < 0.05$ vs. normal serum). *C,* an RCC patient serum sample (1:1 dilution in complete medium) previously determined by ELISA to contain elevated levels of TNFα was incubated for 72 h with T cells from either the corresponding RCC patient or from a healthy donor, at which time, the cells were assessed for apoptosis by TUNEL. One aliquot of patient serum was first treated with anti-TNFα antibodies to neutralize the cytokine present in the sample. Cells treated with TNFα alone or with GM1 plus TNFα served as controls. *Columns,* mean of three experiments; *bars,* SE (*, $P < 0.001$; **, $P < 0.01$ vs. media alone; ***, $P < 0.05$ vs. normal serum). *D,* resting peripheral blood T-cells isolated from three patients with RCC were incubated *in vitro* with their own respective sera (1:1 dilution in complete medium) for 72 h prior to being assessed for apoptosis by trypan blue exclusion. T cells from three healthy donors treated with autologous sera served as negative controls. *Columns,* mean of patient sera from three experiments done in triplicate; *bars,* SE (*, $P < 0.001$ vs. normal serum).

with the SK-RC-45_{hi} tumor line (Fig. 3*C*), TILs and peripheral blood T cells from RCC patients are already coated with the ganglioside upon isolation (Fig. 6*A*). These results suggest that elevated levels of circulating GM1 exist in these patients, consistent with our finding that explanted, primary RCC tumors stain brightly for GM1 with FITC-labeled cholera toxin (Fig. 3*A*). Our results, demonstrating the close positive correlation between TNFα and ganglioside production by RCC lines (Fig. 1*A* and *B*), suggested the notion that patients bearing intensely GM1-positive RCC tumors would also have elevated serum TNFα. To assess this possibility, serum samples from 11 RCC patients with metastatic disease, 8 RCC patients with localized disease, and 6 healthy controls were measured for TNFα content by ELISA. The results of this analysis, presented in Fig. 6*B*, indicated that the molecule was essentially undetectable in the normal serum (0.2 pg/mL), varied in concentration from 1 to 10 pg/mL in the sera of patients with localized disease, and ranged between 4 and 34 pg/mL in the sera of patients with metastatic disease.

Resting T cells from a healthy donor and from a patient with metastatic disease were also compared for their susceptibilities to TNFα, GM1, or both agents in synergistic combination, as well as to metastatic patient serum previously shown to contain elevated levels of TNFα. As compared with the T cells from a normal donor, which exhibited sensitivity only to the positive control treatment of GM1 plus TNFα, but not to either rTNFα or RCC patient serum alone, the T cells from the metastatic RCC patient were highly susceptible to both TNFα and to patient serum, unless the latter was first treated with anti-TNFα antibodies (Fig. 6*C*). These results differ from those obtained with serum from an individual with localized disease, which had minimal levels of TNFα, and hence, could not kill the patient T cells (data not shown). Finally, unlike three normal sera, which exhibited no apoptogenic effect on coincubated autologous normal T-cells (averaged, *line in* Fig. 6*D*), each of three metastatic patient sera were able to induce the apoptosis of peripheral blood T-cells from the corresponding individual, the percentage of T cells killed correlating directly with the TNFα content of the serum, and varying from 28% to 42% of the target cell population (Fig. 6*D*). Collectively, these data reflect the fact that the patient T cells, but not healthy T cells, were coated with GM1 derived from the tumor, and hence, were sensitive to either rTNFα or to the TNFα present in the RCC patient serum.

Discussion

Here, we show that multiple RCC-derived molecules can act in synergy to mediate the apoptotic death of T lymphocytes: RCC-induced T-cell apoptosis can be substantially inhibited if the tumor cell/T-cell coincubations are performed with either ganglioside-depleted tumor cells, or in the presence of neutralizing anti-TNFα antibodies. The notion that there is a synergistic interaction between tumor-derived gangliosides and TNFα for inducing T-cell apoptosis is further corroborated by studies with recombinant TNFα and a purified form of the overexpressed RCC ganglioside GM1 (17), which shows similar cooperativity for killing T cells when used together *in vitro*.

Previous studies from our laboratory indicated that RCC cell lines stimulate the apoptosis of Jurkat cells and T lymphocytes by a mechanism that could be largely inhibited by the glucosylceramide synthesis inhibitor 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (7), an indication that tumor gangliosides were involved. This inhibition was maximal when the tumor cells were pretreated with the ganglioside synthesis inhibitor for 5 days prior to initiating the coculture experiment, a time frame consistent with that required for maximal reduction of tumor ganglioside synthesis, as assessed by HPTLC (33). In this study, the substantial resistance of caspase-8–negative Jurkat cells to a ganglioside-shedding RCC tumor line provided the first suggestion that multiple tumor products might be synergizing to induce T-cell death. The fact that the expression of caspase-8, the apical molecule in the receptor-dependent apoptotic pathway (32), is requisite for maximum lymphocyte susceptibility to RCC-mediated apoptosis, indicates that the renal tumor cells mediate at least some components of their apoptogenicity through a death ligand. In this regard, TNFα was hypothesized to be an RCC-associated death ligand potentially involved in tumor-induced T-cell apoptosis. This is because mutations in the VHL tumor suppressor protein are common in clear cell RCC (21), and lead to dysregulated synthesis of a number of proteins, including TNFα (23). Indeed, reporter gene assays confirm that TNFα mRNA is repressed by the wild-type VHL gene but is actively translated in the VHL mutants (23). These findings are consistent with Northern blot analyses, RT-PCR, and immunohistochemical studies, which all indicate that, unlike normal kidney, human kidney cancer cells themselves constitutively synthesize TNFα (34). Because in addition to the TNFα generated by VHL-mutated tumor cells, a significant bolus of RCC-associated TNFα also likely derives from macrophages that so commonly infiltrate those tumors *in vivo* (35), it is probable that the renal tumor environment typically contains high levels of TNF. Supporting this notion is the fact that sera obtained from RCC patients assessed in our study had highly elevated levels of TNFα as compared with undetectable levels present in the sera from healthy volunteers.

It is interesting to note that whereas RCC lines elaborating both gangliosides and high levels of TNFα could efficiently mediate the apoptosis of resting T cells, activated T cells can readily be killed by an RCC line synthesizing gangliosides alone. This likely relates to the fact that whereas both resting and stimulated T cells express TNFR1, only the activated lymphocytes express TNFα. We would thus postulate that SK-RC-45_{hi}–derived TNFα efficiently kills resting T cells in conjunction with tumor-derived gangliosides, although SK-RC-45_{low} (the RCC line synthesizing GM1 but only low levels of TNFα) kills activated T cells by virtue of a synergistic interaction between tumor-derived gangliosides and T cell–derived TNFα. Similar findings hold true for the purified reagents: efficient apoptosis of resting T cells requires treatment with both GM1 and TNFα, whereas activated T cells can be killed by a low dose of GM1 alone.

NFκB is a transcription factor whose activity is a requisite for the *de novo* synthesis of numerous antiapoptotic proteins (36). Because previous studies indicated that RCC gangliosides inhibit PMA/ionomycin–induced NFκB activation (11, 12, 20), we hypothesized that ganglioside-mediated sensitization of T cells to TNF-induced activation of the caspase cascade (37) might be a mechanism for the observed synergy. Consistent with this thought are the results presented here indicating that RelA overexpression protects T-cell targets from ganglioside/TNFα-induced apoptosis, and that the ability of TNFα to kill Jurkat cells that are already NFκB-inhibited (by a transgene encoding the IκBα super repressor) cannot be further enhanced by pretreatment with GM1.

The importance and *in vivo* relevance of these studies is amplified by our finding that peripheral blood T-cells isolated from patients with RCC stain much more strongly for GM1 than do the T cells isolated from healthy controls. That this enhanced GM1 staining on T cells derives from tumor-shed gangliosides is suggested by two additional observations: (*a*) when stained with FITC-labeled cholera toxin, tumor cells from eight of eight resected RCC expressed highly elevated GM1 levels as compared with the negligible levels characterizing NKE cells; and (*b*) when cultured *in vitro* with either GM1-overexpressing RCC tumor cells or their cell-free, spent culture supernatants, T cells from healthy donors became coated with the ganglioside. Other experiments reported here showed that, functionally, the strong GM1 positivity of patient T cells correlated with their susceptibility to apoptotic death if treated *in vitro* with either TNFα or with patient serum, the latter's toxicity is based on its elevated TNFα concentration, as indicated both by ELISA and the ability of anti-TNFα antibodies to inhibit the effect.

These studies thus elucidate a novel mechanism by which RCC may be enhancing its own growth and metastatic expansion while combining previous, seemingly unrelated, anecdotal observations into a unified hypothesis defining the means by which renal tumors may be killing T cells. Earlier reports from a variety of laboratories described a role for death receptor–based apoptotic schemes in mediating T-cell death, but definitive tumor-associated ligands have remained elusive and uncertain (38). Other laboratories characterized gangliosides as overexpressed, tumor-associated immunosuppressive molecules (39–41), somehow involved in tumor progression and metastasis (42, 43), yet the mechanism by which these glycosphingolipids produced their deleterious effects on the immune system were obscure. Our laboratory did show that RCC gangliosides could suppress NFκB activation in T cells as well as their downstream synthesis of NFκB-dependent antiapoptotic proteins (6, 7, 11, 12, 20), but the subsequent events or "second signal" that could render lymphocytes apoptotic was undetermined. In the series of experiments described here, we now show that tumor-derived gangliosides can sensitize T cells to TNFR1-mediated apoptosis, induced by TNFα present in the RCC tumor microenvironment. The fact that numerous histologically distinct tumors both overexpress gangliosides and are infiltrated by macrophages, suggests the possibility that this synergistic mechanism of tumor-induced T-cell apoptosis may extend to many forms of cancer. Interestingly, a role for RCC-associated TNFα in tumor-induced T-cell apoptosis is consistent with the reported efficacy of infliximab in a phase II clinical trial, in which the anti-TNFα antibodies slowed or stabilized disease in ∼32% of RCC patients with advanced disease (44). In a second, more recent phase II, trial, 46% of RCC patients entering the study with progressive disease obtained a clinical benefit from infliximab therapy, and at higher doses of the anti-TNFα antibody, the clinical benefit increased to 61% of patients (45). These results collectively imply that agents which neutralize tumor-associated TNFα or block ganglioside synthesis might enhance both spontaneous antitumor immune responses and the efficacy of immunotherapeutic interventions. Current studies are directed at defining the mechanism by which gangliosides inhibit NFκB activation, and determining whether TNFα and gangliosides synergize through additional pathways to mediate T-cell killing.

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