Addressing the “Fas Counterattack” Controversy: Blocking Fas Ligand Expression Suppresses Tumor Immune Evasion of Colon Cancer In vivo

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Abstract

Fas ligand (Fasl/CD95L) is a transmembrane protein belonging to the tumor necrosis factor superfamily that can trigger apoptotic cell death following ligation to its receptor, Fas (CD95/APO-1). Expression of FasL may help to maintain tumor cells in a state of immune privilege by inducing apoptosis of antitumor immune effector cells—the “Fas counterattack.” However, the ability of FasL to mediate tumor immune privilege is controversial due to studies that indicate FasL has both pro- and anti-inflammatory activities. To resolve this controversy and functionally define the role of FasL in tumor immune evasion, we investigated if suppression of endogenously expressed Fasl in colon tumor cells resulted in reduced tumor development and improved antitumor immune challenge in vivo. Specifically, Fasl expression in CMT93 colon carcinoma cells was down-regulated following stable transfection with a plasmid encoding antisense Fasl cDNA. Down-regulation of Fasl expression had no effect on tumor growth in vitro but significantly reduced tumor development and improved antitumor immune challenge in vivo. Tumor size was also significantly decreased. Reduced Fasl expression by tumor cells led to increased lymphocyte infiltration. The overall level of neutrophils present in all of the tumors examined was low, with no difference between the tumors, irrespective of Fasl expression. Thus, down-regulation of Fasl expression by colon tumor cells results in an improved antitumor immune challenge in vivo, providing functional evidence in favor of the “Fas counterattack” as a mechanism of tumor immune evasion.

Introduction

Tumor escape is a major obstacle to successful immunotherapy (1). Despite compelling evidence that immunogenic tumors can be rejected by the immune system under optimum conditions, a large number of tumors continue to grow and evade immune-mediated elimination. During carcinogenesis, tumors develop multiple mechanisms to evade the host immune response. Up-regulation of Fas ligand (Fasl/CD95L) expression may represent one such mechanism. Fasl was first identified in 1993 as a 40 kDa type II transmembrane protein belonging to the tumor necrosis factor (TNF) family (2). Fasl interacts with its receptor, Fas (CD95/APO-1), and can trigger a cascade of subcellular events culminating in the apoptotic cell death of sensitive cells (3). This interaction plays an important role in immune homeostasis (4) and in the maintenance of immune privilege in sites such as the eye (5) and testis (6). In these sites, constitutive expression of Fasl may limit inflammatory responses and maintain relative immunosuppression by inducing apoptosis in infiltrating proinflammatory lymphocytes.

The discovery that Fasl is also expressed by a variety of tumor cells raised the possibility that Fasl may mediate immune privilege in human tumors (7–9). Activated T cells express Fas and are sensitive to Fas-mediated apoptosis (10). Thus, up-regulation of Fasl expression by tumor cells may enable the tumor cells to kill infiltrating antitumor lymphocytes. Numerous studies have shown that tumor-expressed Fasl is capable of killing Fas-bearing, sensitive cells in vitro (7, 9), whereas expression of Fasl by human tumors is associated with apoptosis and loss of tumor-infiltrating lymphocytes (TIL) in vivo (11, 12).

However, the role of Fasl in tumor-mediated immunosuppression is controversial. In addition to its well-defined role in apoptosis, Fasl/Fas interactions may mediate other responses such as cytokine secretion and inflammation. In fact, genetically engineered overexpression of Fasl in allografts of tissues and tumor cells in many instances targeted these cells for rapid rejection in vivo (13–15). Rejection was mediated primarily by neutrophils. These findings contrast with others showing an immunoprotective effect of Fasl (16–18). Reasons proposed to account for these discrepancies include differences in the level of Fasl expressed by the allografts (17, 19), sensitivity of the transduced cells to apoptosis (20), overexpression of membrane-bound Fasl (21), and/or the moderating effects of the local microenvironment (22). These studies indicate that Fasl can be either immunoprotective or immunodestructive, and question the ability of Fasl to mediate tumor immune privilege in vivo.

Overexpression of some proteins can lead to atypical consequences. In addition, significant neutrophil recruitment and inflammation has not been observed in tumors endogenously expressing Fasl in vivo. Thus, to functionally investigate the role of Fasl in tumor immune escape and to avoid any potential artifacts of overexpression systems, we investigated whether inhibition of native Fasl expression resulted in reduced tumor development and improved antitumor immune challenge in vivo. In the present study, we specifically inhibited Fasl expression in murine colon tumor cells using antisense RNA. Relative to controls, we found that tumor formation by Fasl antisense–transfected cells was significantly reduced following s.c. injection into syngeneic C57BL/6 mice. In those tumors that did develop, leukocyte infiltration was significantly increased. Interestingly, despite strong expression of...
constitutive FasL in the control tumors, neutrophil recruitment did not occur. Taken together, our results provide for the first time definitive, functional evidence in favor of a role for FasL in tumor immune evasion.

**Materials and Methods**

**Mice.** Four- to six-week-old female C57BL/6 mice were obtained from Harlan UK Ltd. (Oxon, United Kingdom) and maintained in the animal facility of University College Cork. Animal experiments were carried out in accordance with institutional guidelines, using an Animal Research Committee–approved protocol. The mice were maintained in standard environmental conditions with free access to food and water and were allowed to adapt to their environment for 2 weeks before commencement of the experiment.

**Cells and culture conditions.** CMT93, a mouse colon cancer cell line, was kindly provided by Dr. Stephen Todryk (Oxford University, United Kingdom). Cells were maintained in DMEM supplemented with 2 mmol/L glutamine, 100 μg/mL streptomycin, 100 units/mL penicillin, and 10% FCS in a humidified 5% CO₂ atmosphere.

**Generation of stable CMT93 Fasl⁻/⁻/⁻ cell line.** A cDNA fragment complementary, when transcribed, to nucleotides 35 to 371 of the mouse FasL mRNA sequence (GeneBank accession no. U06948) was amplified by PCR using the following set of primers: forward, 5'-GGAGCAGTGACGCTCAGAGTT-3' and reverse, 5'-CCACGGTGAGCCACAGATTG-3'. The fragment spans the translational start site of the mouse FasL gene and was cloned into the SbfI site of pBluescript expression vector (Invivogen, San Diego, CA). Ligation products were transformed into chemically competent XL2-MRF E. coli cells (Stratagene, La Jolla, CA) and analyzed by restriction digestion and automated sequence analysis to determine fragment orientation.

**CMT93 cells were seeded in 24-well plates and transfected the following day using Lipofectamine 2000 reagent (Life Technologies, Inc., Baltimore, CA). Ligation products were transformed into chemically competent XL2-MRF E. coli cells (Stratagene, La Jolla, CA) and analyzed by restriction digestion and automated sequence analysis to determine fragment orientation.

**CMT93 cells were seeded in 24-well plates and transfected the following day using Lipofectamine 2000 reagent (Life Technologies, Inc., Baltimore, MD) according to the instructions of the manufacturer. Briefly, cells were transfected with 1 μg of DNA using 1.5 μL of Lipofectamine 2000 reagent. The medium was replaced 24 hours later with fresh growth media. Blasticidin-supplemented medium (8 μg/mL) was used to select for stable transfectants. Suppression of FasL expression in stably transfected clones was determined by immunoblotting for FasL protein and the clone with the lowest level of FasL was isolated by limiting dilution.

**Immunoblotting.** For immunoblotting, cells were lysed for 1 hour on ice in 20 mmol/L Tris-HCl (pH 7.4), containing 150 mmol/L NaCl and 1% Triton X-100, supplemented with the complete-TM mixture of protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN). Protein concentrations were measured using the bicinchoninic acid protein assay (Pierce, Rockford, IL) according to the instructions of the manufacturer. After removing insoluble material, equivalent amounts of protein (10-50 μg) were loaded onto a 10% polyacrylamide gel, separated by electrophoresis, and transferred onto nitrocellulose membranes. Loading between lanes was assessed by Ponceau-S staining. Membranes were blocked in Blotto (5% skim milk in TBS-0.5% Tween 20) for 1 hour at room temperature. The membrane was then incubated with one of the following primary antibodies: anti-FasL (Ab-1; Oncogene Research Products, Cambridge, MA), anti-Fas (M-20; Santa Cruz Biotechnology, Santa Cruz, CA), anti–TNF receptor 1 (H-5; Santa Cruz Biotechnology), anti–TNF-related apoptosis-inducing ligand (H-257; Santa Cruz Biotechnology), and anti–β-actin (AC-74; Sigma, St. Louis, MO). Primary antibodies were detected with horseradish peroxidase (HRP)–conjugated immunoglobulin G (Dako Corp., Carpinteria, CA) raised against the corresponding species (1:1,000). Peroxidase activity was detected with the enhanced chemiluminescence system (Pierce) and analyzed using Scion Image analysis software (Scion, Inc., Frederick, MD). Changes in Fasl protein expression were determined after normalizing the band intensity of each lane to that of β-actin.

**Flow cytometric cytotoxicity assay.** Cells were plated in triplicate in six-well tissue culture dishes and allowed to reach ~75% confluency as a monolayer. Jurkat T cells were labeled with the red fluorescent dye PKH-26 (Sigma) according to the instructions of the manufacturer. PKH-26 stably incorporates into the plasma membrane, allowing the identification of target cells (23). Aliquots of the target cells (500 μL) were incubated in triplicate for 16 hours with the effector cells at the indicated effector-to-target cell ratios. To block the Fas pathway, neutralizing anti-Fasl monoclonal antibody (MFL3, 10 μg/mL; PharMingen, San Diego, CA) was preincubated on the monolayer for 1 hour at 37°C, followed by the addition of the labeled Jurkat T cells. Cells were harvested, washed in PBS, and stained with Annexin V-FITC (BD PharMingen). The percentage of PKH-26–Annexin V dual positive cells was determined by flow cytometry using a FACScalibur flow cytometer and Cell Quest software (Becton Dickinson, San Jose, CA).

**Detection of soluble Fas ligand.** Soluble Fasl was detected in the culture supernatant of CMT93 cells (transfected and nontransfected) by ELISA. Briefly, after 72 hours, the culture supernatant was removed, centrifuged to remove particulate matter, and analyzed by ELISA according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN).

**Proliferation assays—[³H]thymidine incorporation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.** Cells were seeded at 2 × 10⁵ cells/mL in quadruplicate in 24-well plates and grown for 48 hours. Fresh medium containing [³H]thymidine (1 μCi/mL; Amersham, Buckinghamshire, United Kingdom) was then added to each well. After 6 hours, the cells were washed with ice-cold PBS and harvested for liquid scintillation counting. Alternatively, after 48 hours culture, 20 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) in PBS were added to each well. After 4 hours at 37°C, the medium was carefully removed from the wells and the remaining formazan crystals were dissolved in DMSO. The absorbance at 570 nm was read on a microplate reader.

**PCR.** PCR was done on genomic DNA isolated from individual clones using a DNA isolation kit (Machery-Nagel, Düren, Germany) according to the instructions of the manufacturer. PCR primers were designed using the DNASTAR Lasergene Primerselect programme (DNASTAR, Inc., Madison, WI). Primers were selected which showed no significant homology to any other genes in the European Molecular Biology Laboratory DNA sequence database. Primers were used at a final concentration of 0.1 μmol/L each, deoxynucleotide triphosphates at 50 μmol/L, and MgCl₂ at 1.5 μmol/L. One unit of Taq DNA polymerase was used per 50 μL reaction. The following forward and reverse primers were used, respectively: FasL, 5'-CGGTGGTATTTTTCATGGTTCTGG and CTTGTGATTAGGGCCGTTGTGTT; β-actin, 5'-CCTTCTGGCGATGAACTGTGGCT and GGAACATGTATCTGATCTC.

**Immunohistochemistry.** Tumors were harvested, snap frozen in liquid nitrogen, and fixed in buffered formalin (pH 7.2).

**In vivo tumor growth.** C57BL/6 mice were injected s.c. into the right flank with 3 × 10⁶ tumor cells suspended in 200 μL PBS (n = 25 per group). Five mice from each group were sacrificed each week. Tumor growth was monitored by measuring the width (W) and length (L) of the tumors. The mean tumor diameter was then calculated as W + L/2. After sacrificing, tumors were excised, snap frozen in liquid nitrogen, and fixed in buffered formalin (pH 7.2).
types of cancer, including colon cancer. Neutrophils were identified by immunohistochemical staining for the neutrophil-specific marker lactoferrin (N-20; Santa Cruz Biotechnology) at 0.4 μg/mL. Antibody binding was localized using a biotinylated secondary antibody, avidin-conjugated HRP and 3',3'-diaminobenzidine substrate, contained within the Vectastain avidin-biotin complex detection kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin. When viewed under light microscopy, positive cells stained brown.

To quantify CD45+ TIL infiltration within each tumor, stained tumor sections were observed under light microscopy at ×620. The number of CD45+ cells per 10 high-power fields per section was counted. Results were then expressed as the mean number of CD45+ cells per high-power field. Neutrophil infiltration was assessed in a similar fashion.

**Statistical analysis.** Data are expressed as the mean ± SE. Student's t test or one-way ANOVA was used to compare the mean differences between groups. P < 0.05 was considered significant.

**Results**

**Stable transfection of CMT93 cells with antisense FasL RNA down-regulates Fas ligand.** To examine the role of FasL in tumor immune escape, a 340-bp fragment spanning the translational start site of the mouse FasL gene was generated by reverse transcription-PCR (RT-PCR) and cloned into pBlunt vector in the antisense direction. The positive plasmid was verified by transcription-PCR (RT-PCR) and cloned into pBlast vector. The recombinant plasmids had integrated into the genome of the CMT93 cells (data not shown). The efficacy of FasL antisense RNA in inhibiting FasL protein production was then examined by Western blotting and ELISA, respectively. Western blot analysis revealed a ~90% inhibition in FasL protein expression in clone A3 compared with mock-transfected and untransfected parental cells (Fig. 1B; P < 0.001). This clone was selected for all subsequent experiments. Inhibition of FasL protein expression was specific as the antisense construct did not affect expression of other members of the TNF family, including Fas, TNF-related apoptosis-inducing ligand, and TNF receptor 1 (Fig. 1B). Moreover, FasL antisense RNA significantly decreased the expression of soluble FasL in antisense-transfected cells (CMT93/AS), as determined by ELISA. After 72 hours in culture, there was an almost 2-fold decrease in the amount of soluble FasL present in the supernatant of CMT93/AS cells relative to control cells (P < 0.001; data not shown).

Down-regulation of FasL protein expression on CMT93 cells also substantially inhibited their effector function against Fas-sensitive target cells. There was a marked decrease in the number of apoptotic Jurkat T cells following coculture with CMT93/AS cells, relative to coculture with FasL-expressing mock-transfected (CMT93/C) and untransfected parental cells (Fig. 1C). Cell death induced by CMT93 and CMT93/C cells was confirmed to be FasL-mediated as killing could be blocked by the addition of a neutralizing anti-FasL antibody. In addition, all transfectants, as well as the parental cell line, were found to be resistant to Fas-mediated apoptosis, as assessed by Annexin V staining and flow cytometry following incubation of the tumor cells with 250 ng/mL anti-Fas agonistic antibody (CH-11; Upstate Biotechnology, Charlottesville, VA; data not shown).

Recent studies have shown that Fas can transduce activation and proliferation signals although the mechanism by which this occurs is poorly understood (24, 25). Because CMT93 cells coexpress Fas and Fasl, tumor-expressed Fasl may act in an autocrine or juxtacrine manner to promote tumor growth. However, down-regulation of Fasl expression did not affect the viability and proliferation rate of the tumor cells in vitro, as assessed by both MTT assay and tritiated thymidine incorporation (Fig. 1D). Two days after transfection, there was no significant difference in the proliferation rate of the antisense-transfected cells, relative to that of the control cells. However, CMT93/AS tumor cells did retain a low level of expression of the ligand and this low level may be sufficient to transduce any potential proliferation signals.

**Down-regulation of Fasl expression results in reduced tumorigenicity of colon tumor cells.** To investigate whether reduced Fasl expression by colon tumor cells leads to impaired tumor development in vivo, antisense-transfected, parental, or mock-transfected CMT93 cells were injected s.c. into the flanks of syngeneic immunocompetent C57BL/6 mice (3 × 10⁶ cells/mouse).

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**Figure 1.** Fasl antisense RNA suppresses Fasl expression by CMT93 cells. A. Fasl expression was detected in CMT93 cells at both the mRNA level and protein level by RT-PCR and Western blotting, respectively. Expression of the housekeeping gene β-actin was used as an internal control. B. Immunoblot analysis of Fasl protein expression revealed a reduction in Fasl protein levels in Fasl antisense–treated CMT93 cells (CMT93/AS), relative to parental (CMT93) and mock-transfected (CMT93/C) cells. C. CMT93, CMT93/C, or CMT93/AS cells were incubated in triplicate at the indicated effector-to-target cell ratios with fluorescently labeled Jurkat cells in the presence or absence of a neutralizing anti-Fasl antibody (MFL3). CMT93/AS cells displayed reduced cytotoxicity against Fasl Jurkat target cells. D. Growth rates of CMT93, CMT93/AS, and CMT93/C cells were analyzed by both tritiated thymidine incorporation and MTT assay 48 hours after seeding. Proliferation is expressed relative to untransfected control cells. Columns, mean of three independent experiments; bars, SE.
To follow the course of tumor development, five mice from each group were randomly selected and sacrificed every 7 days for a total of 5 weeks and any tumors present excised. Tumors were found in a total of 30 mice. However, the rate of tumor formation was significantly reduced in mice injected with FasL-low/negative (FasL\textsuperscript{Low/−}) CMT93/AS cells (Fig. 2A). Examination of mice sacrificed at week 5 revealed that none of the mice injected with FasL\textsuperscript{Low/−} CMT93/AS cells developed tumors, whereas three of those injected with FasL-positive (FasL\textsuperscript{+}) CMT93/C cells and four injected with parental cells developed tumors. In fact, overall, there was a 2.3-fold decrease in the number of tumors derived from FasL\textsuperscript{Low/−} CMT93/AS cells compared with CMT93/C cells (P = 0.0165)—in total, 6 tumors developed in mice inoculated with CMT93/AS cells compared with 14 in those inoculated with CMT93/C tumor cells.

Moreover, the size of the tumors derived from FasL\textsuperscript{Low/−} CMT93/AS cells was significantly reduced relative to FasL\textsuperscript{+} CMT93/C and parental CMT93 cells (P = 0.0033). Analysis revealed that tumors derived from CMT93/AS cells were smaller than those derived from CMT93/C and CMT93 parental cells (Fig. 2B). Overall, tumors derived from CMT93/AS cells had an average diameter of just 1.5 mm, compared with those derived from CMT93/C and CMT93 cells which yielded tumors of 2.95 and 3.84 mm, respectively. Thus, suppression of FasL expression by tumor cells resulted in at least a 50% reduction in the average diameter of the tumors which did develop. Together, these findings clearly show that reduction in FasL expression by tumor cells significantly reduces tumor development in mice and favors a role for FasL in tumorigenesis.

Both Western blotting and immunohistochemical analysis confirmed that the levels of FasL protein remained reduced in the CMT93/AS cells \textit{in vivo}. Tumors were excised from all mice and FasL protein levels assessed by both Western blotting and immunohistochemistry. Immunoreactivity for FasL was detected in all excised tumors, but was substantially reduced in the CMT93/AS tumors relative to the parental CMT93 and mock-transfected tumors (Fig. 3).

Reduction in Fas ligand expression by colon tumor cells results in increased infiltration of tumor-infiltrating lymphocytes. To elucidate the mechanism whereby down-regulation of FasL expression by tumor cells results in reduced tumor development \textit{in vivo}, we assessed the level of lymphocyte infiltration in the excised tumors. Activated lymphocytes express Fas and become sensitive to Fas-mediated apoptosis (10). Leukocytes were identified within tumors by immunohistochemical staining for the leukocyte common antigen (CD45) and the number of CD45\textsuperscript{+} cells per 10 high-power fields per tumor was determined. As shown in Fig. 4, there was a significant increase in the overall number of...
CD45+ TILs present in FasL\textsuperscript{Low/−} tumors. Analysis revealed a mean 3.7-fold increase in the number of TILs in FasL\textsuperscript{Low/−} CMT93/AS tumors relative to FasL\textsuperscript{+} CMT93/C tumors (\(P = 0.0072\)). These results indicate that tumor-expressed FasL has an inhibitory effect on infiltration of TILs and are consistent with other studies demonstrating that FasL contributes to immune privilege in human tumors (26, 27).

Constitutively expressed Fas ligand does not trigger the recruitment of neutrophils. Given the studies showing that overexpression of recombinant FasL in allografts and tumor cells can trigger an inflammatory response and rejection of the grafts, we determined whether constitutively expressed native FasL triggers neutrophil infiltration \textit{in vivo}. Neutrophils were identified based on their characteristic multi-lobed nuclei, together with immunohistochemical staining for lactoferrin. Both parental and mock-transfected CMT93 cells constitutively express FasL; however, histologic analysis revealed a lack of inflammation and neutrophil recruitment in response to tumor-expressed FasL (Fig. 5). Moreover, there was no difference in the level of neutrophil infiltration between tumors that expressed FasL and FasL\textsuperscript{Low/−} tumors. These findings are consistent with a role for tumor-expressed FasL in inducing apoptosis rather than activation or recruitment of neutrophils. Indeed, activated neutrophils have previously been shown to be sensitive to Fas-mediated apoptosis \textit{in vitro} (28).

Discussion

The role of FasL in tumor immune evasion and immune privilege is controversial (29, 30). Despite compelling evidence that tumor-expressed FasL is associated with immune evasion, attempts to enhance allograft survival by overexpressing FasL revealed a potential proinflammatory activity for this death ligand, and suggested a more complex biology than previously suspected. However, overexpression of some proteins can lead to atypical consequences. Thus, in an effort to functionally investigate the role of FasL in tumor immune evasion and to avoid any potential artifacts associated with overexpression of FasL, we decreased FasL protein expression in mouse colon tumor cells that constitutively express FasL. If FasL expression promotes tumor immune privilege, then lack of FasL would be expected to result in decreased tumor immune evasion. This, in turn, should lead to reduced tumor formation and growth due to an improved antitumor immune challenge \textit{in vivo}.

In the present study, we found that expression of FasL antisense RNA resulted in a \(\sim\) 90% reduction in FasL protein in CMT93 colon tumor cells. Soluble FasL production was also decreased. S.c. injection of these FasL\textsuperscript{Low/−} tumor cells into immunocompetent mice resulted in impaired tumor formation relative to FasL\textsuperscript{+} nonspecific transfected control cells (\(P = 0.0165\)). Tumor size was
The increased infiltration of TILs seen in FasL\textsuperscript{Low/−} target cells following coculture with FasL\textsuperscript{+} tumor cell lines (7, 8). activated T cells are sensitive to Fas-mediated apoptosis (10). due to decreased apoptosis of the TILs. TILs express Fas (31) and done \textit{in vitro} origins reported to express FasL (8, 32–34). Functional studies of immune evasion and immune privilege includes the extensive survival of the tumor. This shows that functional FasL expression, the reduction of tumor-expressed FasL and resulted in decreased tumor formation. This shows that functional FasL expression, combined with loss of sensitivity to Fas-mediated apoptosis, provides malignant cells with a potent mechanism with which to evade the immune system, promoting the development and survival of the tumor.

Additional evidence supporting a role for Fasl in tumor immune evasion and immune privilege includes the extensive number of tumors and tumor cell lines of varying histologic origins reported to express FasL (8, 32–34). Functional studies done \textit{in vitro}, as well as evidence from \textit{in situ} studies, indicate that Fasl expressed by tumors is functional and able to induce apoptosis of lymphocytes present in the tumor microenvironment. Studies have also shown that disease progression and metastasis is associated with progressively increased expression of FasL (35, 36). Numerous animal studies provide further evidence demonstrating the ability of tumor-expressed Fasl to down-regulate antitumor immune responses. S.c. injection of Fasl-expressing murine melanoma cells into Fas-deficient \textit{lpr} mutant mice resulted in delayed tumor growth compared with wild-type mice (37). In addition, Fasl-transfected tumor cells triggered the depletion of natural killer cells (38) and an impaired antibody response (39) following injection into mice. Moreover, transgenic expression of Fasl on thyroid follicular cells or stable transfection of colon tumor cells with Fasl resulted in a strong inhibition of the allospecific cytotoxic response and either a reduction or a shifting of the alloantibody response toward a Th2 antibody response (16, 39). The ability of Fasl to promote tumor immune escape in the face of an active, specific immune response was also shown recently using mice transgenic for the rat HER-2/neu oncogene (NEU-TG; refs. 18, 40). These mice develop spontaneous breast tumors after a first pregnancy. Following immunotherapy, some rNEU-TG mice were found to develop late “escape” tumors despite the presence of an rNEU-specific immune response. Characterization of these escape tumors revealed that they continued to express rNEU but had acquired constitutive FasL expression, which was associated with apoptosis of infiltrating T lymphocytes \textit{in situ}. Thus, together, these results provide compelling evidence in favor of a role for tumor-expressed Fasl in immune evasion.

Much of the controversy surrounding the “Fas counterattack” as a mechanism of tumor immune evasion stems from transplantation studies demonstrating that forced overexpression of Fasl in transplants induced accelerated destruction or rejection of the transplanted organs via recruitment of neutrophils. As a mediator of immune privilege, it was thought that Fasl could be used in transplantation biology to control rejection. Indeed, prolonged survival of allografts was reported in some studies following Fasl gene transfer to organs such as thyroid (19) and kidney (41). In contrast, many other studies reported rigorous inflammation and accelerated rejection following transplantation of Fasl-overexpressing allografts (14, 15, 42). However, examination of our tumor specimens revealed that constitutively expressed Fasl is not proinflammatory. Not only was there a lack of neutrophil infiltration in response to native Fasl, but there was also no difference between the tumors, regardless of Fasl expression. Whereas several studies have shown that Fas/Fasl ligation may stimulate the secretion of proinflammatory cytokines and chemokines including interleukin (IL)-6, MCP-1, and IL-8 in various cell types (43, 44), results from the current study suggest that excessive inflammation and neutrophil recruitment are directly a result of enforced, ectopic, expression of Fasl in cells which do not endogenously express the ligand. Endogenous expression of Fasl \textit{in vivo} is regulated by a variety of processes. Although synthesized as a transmembrane protein, Fasl can be cleaved by a specific metalloproteinase, matrix metalloproteinase-7, to form soluble Fasl (45). Recent findings suggest that the membrane-bound form of Fasl is the major proinflammatory form of Fasl, whereas soluble Fasl is noninflammatory and in fact opposes the proinflammatory activity of membrane-bound Fasl (21). Thus, the membrane-to-soluble Fasl ratio may be of vital importance in determining the effect of Fasl \textit{in vivo}. Enforced expression of Fasl may bypass the normal regulatory controls for expression of this molecule, tipping the balance in favor of inflammation. Too little soluble Fasl would ensure that any proinflammatory effects of membrane-bound Fasl would dominate. Indeed, CMT93 cells express both forms of Fasl and are not subject to neutrophil-mediated rejection \textit{in vivo}.

In addition, studies have revealed that resident tissue macrophages are highly susceptible to Fasl-induced apoptosis and the apoptotic demise of these cells preceded neutrophil infiltration (46). Thus, the sudden introduction of large numbers of Fasl-overexpressing cells in mice may trigger excessive apoptosis of macrophages present in the allograft microenvironment and the production of proinflammatory cytokines or chemokines. In contrast, selective pressure during the gradual evolution of a spontaneous tumor would ensure that Fasl up-regulation would only occur where it would be advantageous to the tumor. Indeed, our study shows that the physiologic level of Fasl found in tumor cells which endogenously express Fasl does not induce neutrophil infiltration but is still sufficient to delete antitumor lymphocytes.

Furthermore, in sites of immune privilege, Fasl does not act in isolation (47). In the eye, Fasl represents only one of at least eight independent mechanisms that function to preclude any potentially destructive inflammatory responses (48). In addition, the tumor microenvironment is an immunosuppressive one. Tumors can produce a variety of immunoinhibitory cytokines, including transforming growth factor \(\beta\), IL-10, prostaglandins, and gangliosides, which down-regulate the activity of antitumor immune cells. In such an environment, the anti-inflammatory and immune down-regulatory activities of tumor-expressed Fasl may be favored.

In conclusion, our results provide for the first time direct \textit{in vivo} evidence that tumor-expressed Fasl contributes to tumor immune evasion. Low levels of Fasl led to increased infiltration of TILs and reduced tumor formation and growth.
We have also shown that FasL expression by tumor cells is not proinflammatory. On the contrary, native FasL expression by tumor cells is advantageous to tumor development. Because FasL is a major inhibitor of antitumor responses, disarming the "Fas counterattack" may tip the tumor-immune balance in favor of the immune system and ultimately result in improved anticancer therapies.

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