Loss of Functional Fas Ligand Enhances Intestinal Tumorigenesis in the Min Mouse Model

Barbara Fingleton, Kathy J. Carter, and Lynn M. Matrisian

Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, Tennessee

Abstract

Fas ligand (FasL/CD95L), a member of the tumor necrosis factor family, interacts with a specific receptor Fas, ultimately leading to cell death. Tumor expression of FasL has been proposed to aid in immune evasion through a "Fas counterattack" mechanism but has also been described as a proinflammatory factor. Here, we tested the role of FasL in a mouse model of spontaneous tumor development. We used the Min mouse in which multiple benign polyps develop in the intestine due to a mutation in the *Apc* tumor suppressor gene. Mutant mice deficient in functional FasL, termed *gld/gld*, were crossed to Min mice to generate tumor-prone animals lacking functional FasL. Comparison of FasL-deficient versus proficient Min mice revealed a significant increase in polyp number in the *gld/gld* mice. We next assessed immune cell infiltration into adenomas. There was no difference in the number of either lymphocytes or macrophages; however, the number of tumor-infiltrating neutrophils was 3-fold lower in the *gld/gld* specimens compared with controls. Neutrophil migration *in vitro* was stimulated by wild-type but not mutant FasL. In a nontumor-bearing colitis model *in vivo*, neutrophil recruitment to the intestine was also reduced in *gld/gld* mice. Although the Fas counterattack hypothesis suggests that the absence of FasL would result in increased immune-mediated tumor elimination, the opposite is true in the Min model with lack of functional FasL associated with reduced neutrophil influx and increased tumor development. Thus, the proinflammatory rather than counterattack role of tumor FasL is more relevant. [Cancer Res 2007;67(10):4800–6]

Introduction

Fas ligand (FasL/CD95L) is a type II transmembrane protein that can also be found in a soluble form through the action of metalloproteinases (1–3). Its expression was initially associated with lymphoid cells where it is important in maintaining T-cell homeostasis through a process known as activation-induced cell death (4). FasL expression has also been described in epithelial cells, particularly in sites of immune privilege. The receptor for FasL, Fas, is ubiquitously expressed (5). Canonical signaling through the Fas receptor triggers the formation of the death-induced signaling complex that results in activation of the proximal caspase caspase-8 and subsequent activation of a caspase cascade, ultimately leading to execution of the apoptosis program (6). Mice with a spontaneous mutation of the *FasL* gene have been described (7). Originally, the mutated gene was unidentified and the mice were named for their phenotype of generalized lymphoproliferative disorder (gld; ref. 8). The phenotype of enlarged thymus and lymph nodes as well as splenomegaly is due to an inability to remove lymphoid cells, and the resulting autoimmune disease leads to animal death at ~6 months of age. Once the target gene was identified, the nature of the mutation was revealed. In the *gld* allele of FasL, there is a point mutation that changes the amino acid phenylalanine to leucine, thus abrogating ligand-receptor binding (7, 9). Hence, mice carrying the mutation express FasL; however, it is nonfunctional as it is unable to bind Fas.

Expression of FasL is widely reported in tumor cells where it has been suggested as a mechanism of counteracting immunosurveillance (10–13). The "Fas counterattack" is a controversial hypothesis whereby FasL on the surface of tumor cells ligates Fas receptor expressed by infiltrating lymphocytes, leading to their death and thus disarming of the host immune response (11, 14). Additional roles for FasL have been described (e.g., as a chemotactic molecule; refs. 15–18). Such a role would be expected to have an opposite result to the Fas counterattack, whereby enhanced infiltration of tumors by immune cells may more efficiently clear a nascent tumor.

$Apc^{Min/+}$ is a mouse model that resembles the human familial adenomatous polyposis syndrome (19). In this mouse model, a mutation at residue 850 in the *Apc* gene was induced and selected during a random ethylnitrosourea mutagenesis screen (20). APC is a tumor suppressor and regarded as a gatekeeper for intestinal tumorigenesis. Mutations in APC or other protein components of an APC complex prevents degradation of β-catenin, leading to its nuclear accumulation and expression of Wnt target genes. Ultimately, this results in the development of numerous benign polyps predominantly in the small intestine. Progression to malignancy is not usually observed in $Apc^{Min/+}$ mice as animals become moribund due to occlusion of the intestinal tract and development of anemia.

Our goal was to use this spontaneous model of early tumor development to examine a role for the Fas counterattack in the genesis and outgrowth of $Apc^{Min/+}$ tumors. The Fas counterattack hypothesis proposes that tumor FasL expression is responsible for destruction of any infiltrating immune cells, thus allowing unchecked outgrowth of the tumor. We therefore expected that tumor cells in the animals carrying only nonfunctional *gld* alleles of FasL would not be able to launch an effective counterattack and hence would be destroyed by patrolling immune cells. Instead, we observed higher tumor frequency in the gldxMin animals compared with control Min animals with no changes in variables, such as proliferation or tumor apoptosis. This correlated with reduced infiltration of the tumors by neutrophils and suggests that the chemotactic activity of FasL is of relevance in this benign intestinal cancer model.

Requests for reprints: Barbara Fingleton, Department of Cancer Biology, Vanderbilt University Medical Center, 771 PRB, 2220 Pierce Avenue, Nashville, TN 37232. Phone: 615-343-3422; Fax: 615-936-2911; E-mail: Barbara.Fingleton@vanderbilt.edu.

doi:10.1158/0008-5472.CAN-06-4473

Materials and Methods

Animals. All studies were conducted following review and approval by the Institutional Animal Care and Use Committee. *Apc*$^{Min/+}$ and *gld/gld* mice (both on C57BL/6J background) were purchased from The Jackson Laboratory. Male *Apc*$^{Min/+}$ were bred with *gld/gld* females and resulting male *Apc*$^{Min/+}$*;gld/+* were crossed with female *gld/+* to generate *Apc*$^{Min/+}$*;gld/gld, Apc*$^{Min/+}$*;gld/+*, and *Apc*$^{Min/+}$*;+/+* progeny of both sexes that were put on study. Once weaned, all study mice were placed on a high-fat diet (#5015; Purina Mills, Inc.) to enhance tumorigenesis. At 17 weeks of age, study mice were euthanized and the intestines were removed, rinsed in PBS, formalin fixed, and then cut open longitudinally. Polyps were counted, their location was noted, and measure of diameter was obtained by two observers (K.J.C. and B.F.) blinded to the genotype of the specimen. Once counting was completed, polyps were excised and embedded in paraffin. For the microadenoma study, 4- to 5-week-old mice were used. Study mice were euthanized, and intestines were removed and rinsed in PBS. The intestines were cut longitudinally and "jelly rolled" as described previously (21).

Immunohistochemistry. Tissues were fixed in 10% buffered formalin before embedding in paraffin. For FasL localization, anti-FasL (clone A11; Alexis Biochemicals) was used. Microadenomas were identified in jelly rolled 4-week-old intestines by β-catenin immunostaining as has been described (21). Neutrophils were detected using a rat monoclonal anti-neutrophil antibody (AbD Serotec); for macrophages, a rat monoclonal anti-F4/80 antibody (AbD Serotec) was used, whereas for proliferation markers rabbit anti-proliferating cell nuclear antigen (PCNA; Sigma) and Ki67 (DAKO) were used. Apoptosis was assessed using anti-cleaved caspase-3 antibody (Cell Signaling Technology, Inc.), and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis was done with the ApopTag *In Situ* Peroxidase Staining kit (Chemicon). Immunostained slides were counterstained with Mayer's hematoxylin (Sigma). Quantification of staining was achieved by counting the number of positive cells per unit area in multiple high-powered (40×) views of each slide using ImageJ software.

Flow cytometric evaluation of bone marrow leukocytes. Cells were obtained by flushing the marrow cavity of femurs isolated from 9-week-old wild-type (WT) or *gld/gld* C57BL/6 mice with PBS. RBCs were lysed using Pharmalyse (BD Biosciences). Cells (1×10^7) were resuspended in 50 μL aliquots of cold fluorescence-activated cell sorting buffer [1% bovine serum albumin (BSA) in PBS] and incubated for 20 min on ice with anti-CD16/CD32 (BD Biosciences) and then for a further 30 min with fluorophore-labeled antibodies to specific cell surface markers. The antibodies used were Alexa Fluor 488 anti-Gr-1 (BD Biosciences), Alexa Fluor 700 anti-B220 (BD Biosciences), phycoerythrin (PE)-Cy5.5 anti-CD4 (eBioscience), PE anti-CD8 (eBioscience), and PE-Cy7 anti-CD11b (eBioscience). Additional bone marrow samples were incubated with anti-CD45 (AbD Serotec) followed by Alexa Fluor 488 anti-rat antibody. The stained cells were analyzed on a Becton Dickinson LSRII operated through the Vanderbilt flow cytometry core lab.

Isolation and activation of splenocytes. Splenocytes were harvested from 7-week-old male WT or *gld/gld* C57BL/6 mice using a method described previously (2). Single-cell suspensions were incubated in the presence of 10 μg/mL concanavalin A for 16 h, which was then removed by incubation with 100 μg/mL methyl-α-D-mannopyranoside for 20 min and rinsing in RPMI 1640. Cells were incubated for a further 48 h in RPMI 1640 containing 5 ng/mL recombinant interleukin-2 (Sigma).

To assess antitumor activity of the activated T cells, T cells were incubated with NMuMG murine mammary cells at an E:T ratio of 2:1 for 8 h at 37°C. The T cells were aspirated and the NMuMG monolayers were washed several times with PBS to ensure all T cells were removed. The cells were then incubated for a further 12 h in complete growth medium, after which they were harvested, washed in PBS containing 5 mmol/L EDTA, and fixed in 70% ethanol. Samples were incubated with 50 μg/mL RNase A for 30 min at room temperature before staining with 50 μg/mL propidium iodide. Analysis of DNA content as measured using a BD FACStar flow cytometer was done using FlowJo software.

To assess response of isolated T cells to an apoptotic stimulus, equal numbers (5×10^5) of activated T cells were incubated for 16 h at 37°C with RPMI 1640 supplemented with 100 ng/mL recombinant soluble FasL (Alexis Biochemicals). The cells were then harvested and stained with FITC-Annexin V (R&D Systems). Stained cells were measured using a BD FACStar flow cytometer.

Colitis induction by dextran sodium sulfate. Dextran sodium sulfate (DSS; molecular weight, 36–50,000; MP Biomedicals, Inc.) was used to prepare a 4% solution in water. This was supplied to 8-week-old WT or *gld/gld* male C57BL/6 mice in place of drinking water for 7 days. The mice were weighed daily and checked for bleeding. After 7 days, the animals were euthanized and the colons were excised and flushed with PBS. Small (0.5 mm) pieces were fixed in 10% formalin to enable histologic assessment. The rest of the tissue was minced and incubated for 2 h at 37°C with a freshly prepared tissue digestion solution containing 0.1% (w/v) collagenase (Sigma), 0.025% (w/v) DNase (Sigma), 2% (v/v) dispase (BD Biosciences), and 1% (w/v) BSA (Sigma) in PBS. Following digestion, 1×10^7 cells per sample were incubated with Alexa Fluor 488–labeled anti-Gr-1 antibodies before flow cytometric analysis as described above.

FasL and FasL^{gld} overexpression. Splenocytes from WT and *gld/gld* mice were isolated as described above. Total RNA was extracted using RNeasy (Qiagen). Each RNA (1 μg) was reverse transcribed and the product was PCR amplified using primers for FasL FLAG-tagged at the 5′-end and containing restriction sites for *Bam*H1 and *Hin*dIII at the 5′-end and 3′-end, respectively. The PCR products were subcloned into the pcDNA3.1 expression vector and identity was confirmed by sequencing. Empty vector or vectors containing FasL or FasL^{gld} were transfected using Fugene (Roche) into HCT116 cells [American Type Culture Collection (ATCC)]. Selection of stably expressing cells was achieved using 800 μg/mL G418 (Invitrogen).

Migration assays. Human embryonic kidney 293 cells (3×10^5; ATCC) in regular growth medium [DMEM with 10% fetal bovine serum (FBS)] were plated in each of the lower wells of a 24-well Transwell tissue culture plate (pore size, 3 μm; Costar) and allowed to adhere for 14 h in a 37°C incubator. The medium was then replaced with 0.2 mL/well reduced serum medium (DMEM with 2% FBS) containing 10 μg/mL NOK-2 human FasL neutralizing antibody (BD Biosciences) or isotype control (BD Biosciences) and incubated for 2 h. Meanwhile, neutrophils were isolated from whole blood collected from C57BL/6 mice as has been described (22). Differential staining of the leukocyte population using Hema-Quik II (Fisher Scientific) indicated that ~85% were neutrophils. Leukocytes (1×10^6) in a volume of 0.2 mL serum-free medium were added to the upper chambers of the Transwell system and the plate was incubated at 37°C for 45 min. The filters were fixed in 10% formalin before staining with Giemsa. They were then mounted on slides and the number of cells that had migrated to the undersides of the each filter was counted by microscopic examination of 10 fields per filter under a 40× objective lens. For the HCT116 experiment, cells transfected with vector only, murine FasL, or murine FasL^{gld} as described above were plated in the bottom of the Transwell plates at a density of 5×10^5 per well. After adherence, the medium was changed to include either the K10 murine FasL neutralizing antibody (BD Biosciences) or isotype control at 10 μg/mL as before.

Statistical analysis. Comparisons of differences between two sets of samples were analyzed for significance at the 95% confidence level using Student's *t* test. For analysis of greater than two sets of samples, ANOVA with the Bonferroni post hoc test was used. All analyses were done using GraphPad Prism 4 software.

Results

FasL is widely expressed in Min tumors. Expression of FasL was originally described as being limited to cells of the hematopoietic system (23); however, more recent analysis has suggested that expression is actually more widespread (5), including in normal human intestinal epithelium (24). To determine if FasL is present in the intestinal polyps that arise in *Apc*$^{Min/+}$ mice, we did immunohistochemistry using an anti-mouse FasL antibody (Fig. 1*A*). Multiple polyps from three different

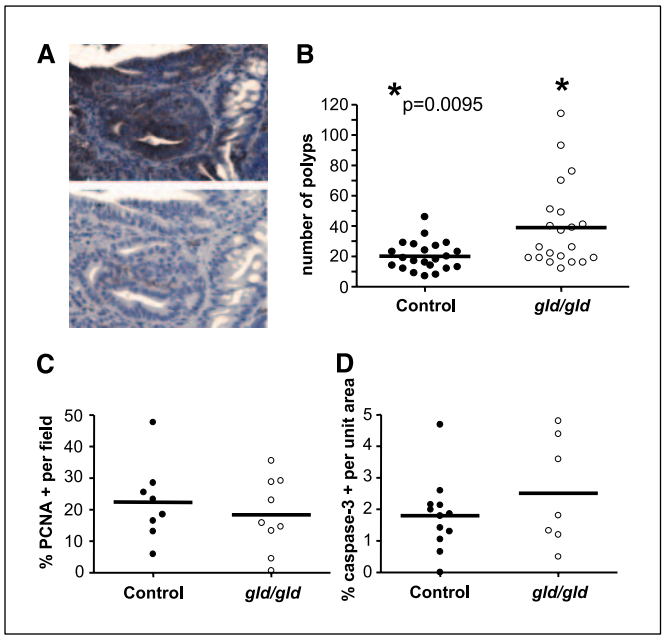

Figure 1. FasL is expressed in intestinal tumors but its removal enhances tumor development. *A,* example of an intestinal adenoma from a 17-wk-old Min mouse showing strong expression (*brown stain*) in epithelial cells. *Bottom,* staining seen with a nonspecific isotype control antibody. Hematoxylin was used as a nuclear counterstain. *B,* tumor number in the small intestine in cohorts of 17-wk-old Min mice either positive (*Control*; $n = 20$) or negative for functional FasL (*gld/gld*; $n = 22$). *C,* proliferation index determined by counting numbers of PCNA-positive cells per total cell number in tumor sections isolated from 17-wk-old Min mice positive (*Control*; $n = 8$) or negative (*gld/gld*; $n = 9$) for functional FasL. *D,* apoptotic index determined by counting numbers of cleaved caspase-3–positive cells per unit area in tumor sections from 17-wk-old Min mice positive (*Control*; $n = 12$) or negative (*gld/gld*; $n = 8$) for functional FasL.

$Apc^{Min/+}$ mice each stained strongly for FasL expression in the intestinal epithelium. The staining in the tumors was significantly more intense than in adjacent normal epithelium. Because FasL was expressed in the tissue and its levels correlated with tumor development, we next used mice deficient in functional FasL (*gld/gld*) to test whether this protein functionally contributed to tumorigenesis.

The number of adenomas is significantly increased in $Apc^{Min/+}$ *gld/gld* mice. Male and female mice all carrying the *Min* allele and WT, heterozygous, or double mutant for the *gld* allele of FasL were maintained on a high-fat diet for 14 weeks after weaning, after which the mice were sacrificed and the intestines were removed. Following rinsing, the small intestines and colons were examined under a dissecting microscope for the presence of visible polyps, taking care not to include Peyer's patches. An initial comparison among the three different FasL genotypes indicated that there was no discernible difference between WT and heterozygous. For this reason, all future comparisons were made between *gld/gld* and controls consisting of WT and heterozygous. After analyzing at least 20 mice in each group, a significant increase in polyp number in the *gld/gld* mice compared with the control group was evident ($P = 0.0095$; Fig. 1*B*). Evaluation of the sizes of the polyps revealed no difference in size distribution between the groups (control = 91.72 $\pm$ 7.5% $\leq$ 2 mm; *gld/gld* = 88.98 $\pm$ 7.2% < 2 mm; $P = 0.36$). As the size was not different, we did not anticipate that there would be differences in proliferation or apoptosis rates; nevertheless, we assessed these using PCNA and Ki67 immunostaining for proliferation and TUNEL and cleaved caspase-3 immunohistochemistry for apoptosis. Neither proliferation nor apoptosis as determined by any variable was significantly different between the two groups (data not shown; Fig. 1*C* and *D*).

Infiltration of inflammatory cells into adenomas. Because FasL is considered of importance in regulation of immune cell homeostasis (4) and potentially immunosurveillance (25), we assessed the degree of infiltration by particular subpopulations of immune cells. This was accomplished by immunohistochemical staining using specific antibodies in multiple tumor sections from both *gld/gld* and controls. There was no difference in macrophage numbers between the two groups (Fig. 2*A*). In addition, contrary to what may be anticipated from the counterattack theory where lymphocytes would be killed by FasL-expressing tumors, there was no significant difference in the number of lymphocytes detected in tumors positive or negative for functional FasL (Fig. 2*B*). In contrast to other leukocytes, neutrophils were found to be differentially present. The number of neutrophils was 3-fold lower in the *gld/gld* specimens in comparison with the control group (Fig. 2*C*). To determine if this difference in neutrophil influx was established from the earliest stages of tumor development, we focused on the microadenoma stage when tumors are just beginning and involve fewer than five intestinal crypts. Tumors at this stage are not obvious and so an immunohistochemical approach was used to detect them. Tumors in $Apc^{Min/+}$ mice are characterized by loss of heterozygosity at the *Apc* locus, resulting in

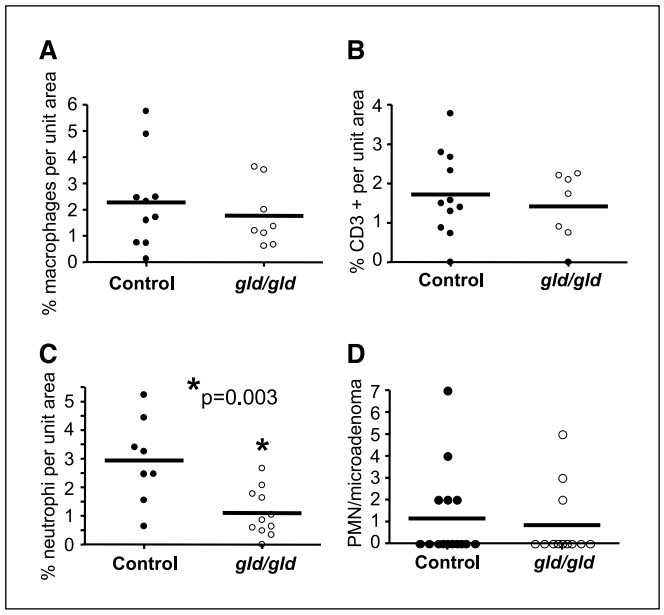

Figure 2. Effects of loss of functional FasL on tumor infiltration by leukocytes and on microadenoma development. *A,* macrophages were immunostained using anti-F4/80 on tumor sections isolated from 17-wk-old Min mice positive (*Control*; $n = 10$) or negative (*gld/gld*; $n = 8$) for functional FasL. Percentage of cells per unit area that were F4/80 positive. *B,* T cells were immunostained using anti-CD3 on tumor sections isolated from 17-wk-old Min mice positive (*Control*; $n = 10$) or negative (*gld/gld*; $n = 7$) for functional FasL. Percentage of cells per unit area that were CD3 positive. *C,* neutrophils were immunostained using antineutrophil on tumor sections isolated from 17-wk-old Min mice positive (*Control*; $n = 8$) or negative (*gld/gld*; $n = 11$) for functional FasL. Percentage of cells per unit area that were neutrophil positive. *D,* neutrophils within microadenomas were counted in serial sections immunostained for β-catenin and neutrophils from jelly rolls of the distal small intestine were harvested from 4- to 5-wk-old Min mice positive (*Control*; $n = 14$) or negative (*gld/gld*; $n = 9$) for functional FasL. Average number of neutrophils per microadenoma calculated after staining a minimum of four lesions per mouse.

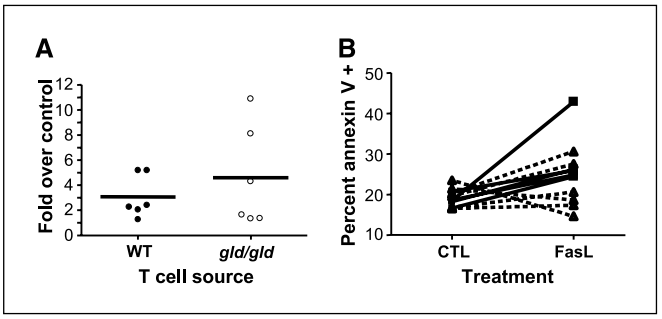

Figure 3. Lymphocytes from *gld/gld* mice maintain cytotoxic activity. *A,* splenocytes from six each WT or *gld/gld* mice were isolated and activated as described in Materials and Methods and incubated at an E:T ratio of 2:1 with the murine tumor cell line NMuMG. The NMuMG cells were harvested and stained with propidium iodide before analysis by flow cytometry. The percentage of cells with sub-G_1 (2N) DNA was calculated for a control culture not exposed to T cells or cultures exposed to T cells from each of the WT or *gld/gld* mice. Fold increase in apoptosis observed in the T-cell–exposed cultures compared with the nonexposed cultures. *B,* activated T cells from six each WT or *gld/gld* mice were incubated for 16 h with recombinant soluble FasL, after which the cells were harvested, stained with FITC-conjugated Annexin V and propidium iodide, and analyzed by flow cytometry. Percentage of apoptotic cells in control, untreated cultures versus those exposed to FasL. *Squares* and *solid lines,* samples from WT mice; *triangles* and *dashed lines,* samples from *gld/gld* mice.

only mutant APC protein that cannot target β-catenin for degradation (26). Hence, β-catenin accumulates in the cells and can be easily detected by immunostaining with a specific monoclonal antibody. Evaluation of the numbers of microadenomas in random fields from either control or *gld/gld* Min mice indicated that the number of early lesions was approximately equivalent between the two groups [0.52 ± 0.65 versus 0.31 ± 0.25 tumors per section; *P* = not significant (NS)]. Analysis of neutrophil number within these early lesions also showed no differences between the genotypes; they were uniformly low (Fig. 2*D*). Hence, the difference in neutrophil influx to the Min adenomas occurs at a later stage than during microadenoma development.

Lymphocytes from *gld/gld* mice maintain tumor-killing activity. Because there were increased numbers of tumors evident in the gldxMin mice compared with control Min mice, we investigated whether this could be attributed to reduced cytotoxic activity of T cells. Splenocytes were isolated from age- and sex-matched WT and *gld/gld* mice. After activation, equal numbers of T cells were added to cultures of an allotypic murine tumor cell line, NMuMG, which we have previously shown to be sensitive to T-cell killing *in vitro* (27). Following a period of coculture, the T cells were removed and the tumor cells were washed, incubated for a further 12 h, harvested, and stained with propidium iodide to determine the percentage of cells undergoing apoptosis. As shown in Fig. 3*A*, the T cells from the *gld/gld* mice did not show a reduced capacity for inducing apoptosis in tumor cells (*P* = NS). It has been previously reported that *gld/gld* T cells show potent cytotoxic activity against renal cancer cells (28), which is mediated predominantly through the perforin/granzyme B pathway.

To further assess the T cells from *gld/gld* mice, we examined their ability to undergo apoptosis. Normally, activated T cells undergo a process of activation-induced cell death that serves to limit circulating numbers of cells following cessation of an immune stimulus (4). In mice deficient for FasL, this process does not occur because FasL is required; however, it has been shown that introduction of adenoviral FasL *in vivo* will induce lymphocyte death in *gld/gld* mice (29). We incubated the activated T cells obtained from WT and *gld/gld* mice with recombinant soluble FasL and assessed levels of T-cell apoptosis using Annexin V staining. As expected, levels of cell death achieved were not extremely high because it is known that the soluble version of FasL is significantly less potent than the transmembrane version (1). Nevertheless, both the *gld/gld* cells and their WT counterparts responded similarly to this relevant apoptotic stimulus (*P* = NS; Fig. 3*B*). Together, these results indicate that the enhanced tumor growth observed in the gldxMin mice cannot be simply attributed to a defective immune response.

FasL, but not the *gld* mutant, causes neutrophil migration *in vitro*. The reduced number of neutrophils in the *gld/gld* versus control Min mice implies that FasL can stimulate neutrophil migration as has been previously suggested (15). To test this, we did a simple migration assay using human embryonic kidney cells as they represent a normal, although immortalized, epithelial cell that expresses FasL protein (Fig. 4*A*). The cells were plated in the lower chambers of a 24-Transwell plate in the presence of human FasL neutralizing antibody or isotype control. Meanwhile, neutrophils were isolated from C57BL/6 mice. Differential staining of the leukocyte population obtained revealed that ~85% were neutrophils (data not shown). The freshly isolated leukocytes were added to each of the upper chambers of the Transwell system. Following incubation, the filters were fixed and stained, after which the number of cells that migrated to the underside of each filter was

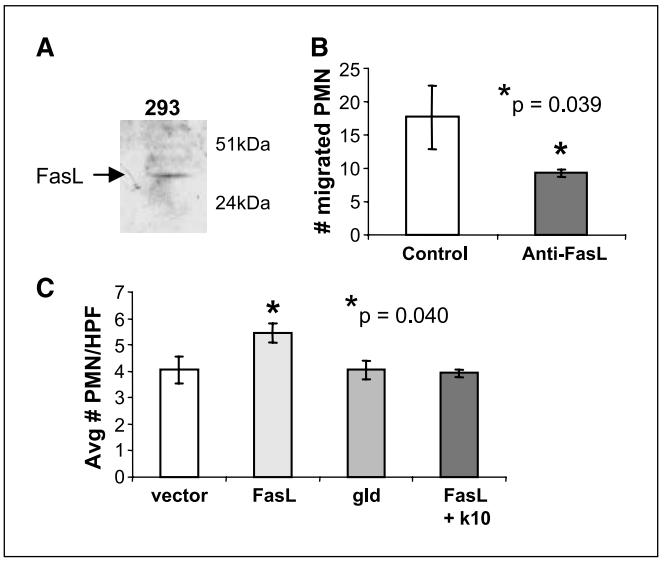

Figure 4. FasL, but not the mutant gld version, induces neutrophil migration *in vitro*. *A,* Western blot showing endogenous expression of FasL (molecular weight, ~43 kDa) in total cell lysate prepared from the human embryonic kidney cell line 293. *B,* polymorphonuclear (*PMN*) cells freshly isolated from murine whole blood were incubated for 45 min at 37°C in the top chamber of a Transwell plate in which 293 cells in the presence of a nonspecific isotype control antibody (control) or the human FasL neutralizing antibody NOK-2 (anti-FasL) had been plated in the lower chamber. Total number of PMN detected by Giemsa staining on the underside of the filters in 10 high-powered views per well (*n* = 3 each condition). *C,* the human HCT116 colon adenocarcinoma cell line was transfected with constructs containing WT murine FasL (*FasL*), gld mutant murine FasL (*gld*), or empty vector (*vector*) and the cells were plated in four or eight (FasL) wells each of the bottom of a Transwell plate. Nonspecific isotype control antibody was added to all wells, except for four that received the mouse FasL neutralizing antibody K10. Freshly isolated murine PMN was added to the top chambers and the plates were incubated for 1 h at 37°C, after which PMN on the undersides of the filters was stained with Giemsa and enumerated in four high-powered fields (*HPF*) per well. Average number of migrated PMN per high-power field for each condition.

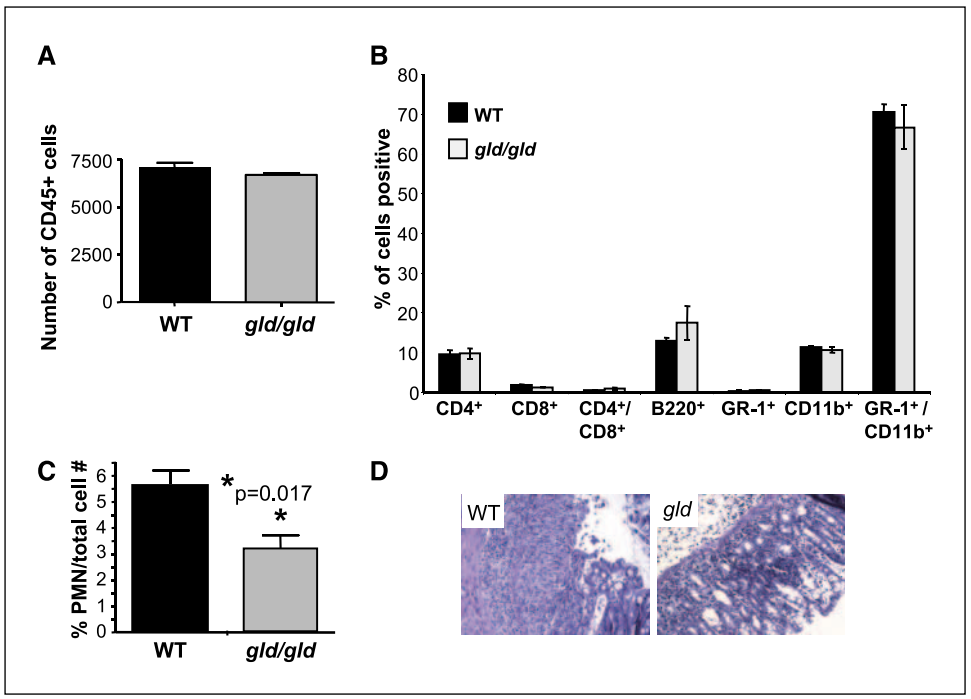

Figure 5. Neutrophil infiltration of colonic tissue is attenuated in the absence of functional FasL. *A,* bone marrow was harvested from 9-wk-old WT (*black*) or *gld/gld* (*gray*) C57BL/6 mice and stained with fluorophore-conjugated antibodies against the T-cell markers CD4 and CD8, the B-cell marker B220, the granulocyte marker Gr-1, and the monocyte marker CD11b. The percentage of total cells that were also positive for each of the other markers is shown for samples from each genotype (n = 3/genotype). *B,* 8-wk-old WT (*black*) or *gld/gld* (*gray*) C57BL/6 mice were placed on a DSS treatment regimen for 7 d, after which the colons were harvested and processed for immunostaining with Alexa Fluor 488–labeled anti-Gr-1 antibody. Percentage of total cells analyzed that were positive for the neutrophil marker (n = 4/genotype). *C,* sample histology (H&E stained) of colon tissue harvested from DSS-treated WT (*left*) and *gld/gld* (*right*) animals.

counted. The presence of the neutralizing antibody almost halved the number of migrated cells (17.7 ± 4.73 versus 9.3 ± 0.58; Fig. 4*B*), indicating that FasL does influence neutrophil migration.

We used the same type of assay to investigate whether the Fas/FasL interaction is required for neutrophil chemoattraction. Full-length WT and *gld* mutant FasL sequences were amplified from splenic RNA of WT and *gld/gld* C57BL/6 mice, respectively. The cDNAs were subcloned into an expression vector and then transfected into the human colonic tumor cell line HCT116. This human colonic adenocarcinoma cell line expresses human FasL. Two clones each of vector-transfected, murine FasL–transfected, and gld-transfected HCT116 cells were plated in triplicate wells of a 24-well tissue culture plate. An extra set of wells was coated with the FasL-transfected clones for neutralization with the K10 anti-mouse FasL neutralizing antibody. The following day, freshly isolated murine neutrophils were put in the upper chambers of the plate and the whole apparatus was incubated for 1 h at 37°C. As before, the number of neutrophils in multiple different high-power fields per filter was counted and the averages were compared. HCT116 cells transfected with FasL showed a statistically significant 25% increase in the number of neutrophils migrated when compared with vector- or gld-transfected clones (Fig. 3*C*). Inclusion of the K10 anti-mouse FasL neutralizing antibody prevented the increased migration. All wells showed a background level of migration likely due to the human FasL protein expressed by HCT116 cells. These data suggest that the neutrophil migration stimulated by FasL is dependent on the ability of the ligand to directly interact with its known receptor Fas on neutrophils.

Neutrophil migration is reduced *in vivo*. To directly test whether the *gld/gld* mutant FasL mice were impaired in neutrophil recruitment as suggested by the *in vitro* assays, we decided to use a direct inflammatory stimulus in the intestine. However, we first needed to exclude the possibility that basal levels of neutrophils were lower in the *gld/gld* mice. We therefore analyzed bone marrow isolated from 9-week-old WT or *gld/gld* C57BL/6 mice for expression of hematopoietic and myeloid lineage-specific cell surface markers using flow cytometry. The markers used were CD45 to determine the overall number of leukocytes in the marrow, CD4 and CD8 for T cells, B220 for B cells, Gr-1 for granulocytes, including neutrophils, and CD11b for monocytes. As can be seen in Fig. 5*A* and *B*, the distribution of the different lineages was equivalent in the presence or absence of functional FasL. Only the $B220^+$ (B cell) and $CD4^+/CD8^+$ (T cell) populations showed increases in the *gld/gld* samples, although these were not significant at this age. Such increases would not be surprising given the lymphoproliferative phenotype associated with the *gld* mutation.

Having established that the *gld* mutant mice were indistinguishable from the WT in terms of neutrophil progenitors, we next tested whether the mobilization of neutrophils into the intestinal tissue was affected in the absence of an oncogenic mutation, such as Apc^{Min}. For this experiment, we used short-term treatment with the inflammation-promoting agent DSS. When DSS is administered in drinking water for 7 days, animals develop an intense inflammatory response within the colorectal region (30). Cohorts of 8-week-old male WT or *gld/gld* C57BL/6 animals were treated with 4% DSS in drinking water for 1 week. We then collected the colonic tissue and used flow cytometry to determine the number of GR-1–positive cells (neutrophils) per total cell population for each mouse. The number of neutrophils within the colonic tissue was significantly reduced in the *gld/gld* samples (Fig. 5*C*). This correlated with a less severe inflammatory response to the DSS treatment as assessed by body weight change (data not shown) and histologic appearance (Fig. 5*D*).

Discussion

Increased expression of a death-inducing protein, such as FasL, in tumors has been regarded as evidence for a tumor-induced antihost response, termed Fas counterattack (31). Here, we have

used a spontaneous model of intestinal tumor development to assess whether the Fas counterattack has a role in the tumorigenic process. We show that, in tumor-prone animals deficient in functional FasL, tumor multiplicity is higher. If the counterattack hypothesis prevailed in this setting, we would predict that lack of epithelial FasL would allow infiltrating T cells to target and remove nascent tumors; thus, tumor number would be lower in the gldxMin mice. In addition, we observed similar numbers of T cells in Min tumors irrespective of the FasL status; however, neutrophil number was decreased in the absence of functional FasL. We then confirmed using both *in vitro* and *in vivo* approaches that expression of the mutant FasL is associated with reduced neutrophil influx. In sum, these data suggest that the chemotactic activity of FasL is more relevant for tumor development than the proposed counterattack, at least in this setting.

Much of the evidence for the Fas counterattack concept comes from correlative studies in which immunostaining of human tumor tissue sections showed FasL-expressing tumor cells juxtaposed to T cells apparently undergoing apoptosis (12, 31, 32). Additionally, *in vitro* experiments where activated T cells were added to cultures of FasL-expressing tumor cells showed induction of apoptosis in the lymphocytes (33). The theory is controversial partly because of technical questions about reagents used, such as antibodies and T-cell lines highly sensitive to Fas-mediated apoptosis (14, 34). There have been several attempts using *in vivo* animal models to functionally test the concept; however, the results have been ambiguous (12, 17, 18, 35, 36). Frequently, researchers have used tumor cells engineered to overexpress FasL and implanted these, usually s.c., in syngeneic mice. In many cases, this has resulted in significant neutrophilic infiltration and rejection of the implanted tumors (17, 18). One article described a neutrophil-independent negative effect on tumor growth (35). Interestingly, these researchers showed that their FasL-transfected tumor cell line (B16 melanoma) was able to induce T-cell apoptosis *in vitro* as suggested by the counterattack hypothesis, but this did not occur *in vivo*. Instead, the FasL-transfected tumors grew significantly more slowly in a manner that was not influenced by neutrophil depletion or by implantation into a Fas-deficient mouse. A recent article attempted to overcome possible artifacts due to overexpression of FasL by instead using a murine colon cancer cell line that endogenously expresses FasL and comparing this with a FasL antisense-transfected version (36). These investigators found decreased growth of the antisense-expressing cells, which was correlated with increased leukocyte infiltration, a result consistent with the Fas counterattack theory. Rather than using allograft models in which a large bolus of tumor cells is introduced into otherwise healthy animals, we chose to examine a spontaneous model of tumor formation. Because there is much evidence to suggest that immune surveillance is affected by a developing tumor systemically as well as locally (37, 38), we rationalized using an endogenous tumor model would give a clearer picture of the true effect of a Fas counterattack. In addition, this model allows for any modifying effects of the intestinal microenvironment that would not be seen in s.c. injection models. This is relevant as microenvironmental factors, such as transforming growth factor-β and extracellular matrix proteins, have been shown to influence the effects of FasL (39, 40). Finally, the $Apc^{Min/+}$ model we have used leads to benign tumor development in contrast to the rather aggressive malignant disease that has been examined previously.

Similar to the counterattack hypothesis, the role of FasL in leukocyte recruitment is also controversial. There is evidence from multiple *in vitro* and *in vivo* experiments that expression of FasL is associated with an inflammatory influx (15, 18, 41–43); however, it is not clear whether FasL is itself the chemotactic stimulus or whether cells induced to die through FasL/Fas interaction then release chemotactic mediators that are directly responsible for leukocyte migration. If FasL itself is the chemotactic signal, then one would predict that the soluble version is the relevant molecule (15, 16). Some *in vitro* studies supporting this have shown chemotaxis of leukocytes in response to increasing gradients of soluble Fas (15, 16). One of the metalloproteinases responsible for solubilization of FasL, matrix metalloproteinase-7 (2, 3), is overexpressed in Min adenomas (44, 45). Therefore, it is possible that soluble FasL is generated in the Min tumors. However, several other investigators have used engineered noncleavable versions of transmembrane FasL and have shown more robust inflammatory responses *in vivo* (42, 46). The noncleavable version is a more effective death-inducing molecule, hence the concept that it is dying cells that induce the leukocyte influx. A key argument against this premise is the idea that apoptosis is regarded as a form of "silent" killing that allows damaged cells to be removed without inducing an inflammatory response. Our *in vitro* experiments would suggest that FasL is itself the chemotactic factor that acts directly on neutrophils. Migration of the neutrophils occurs within less than an hour, which is relatively fast if cell death induction and chemokine production are required. However, these experiments were not designed to specifically test whether FasL is a direct or indirect inducer of neutrophil migration and so the results are not truly informative in this regard. The data from our *in vivo* experiment in which we dosed WT or *gld/gld* mice with DSS to induce colitis also cannot distinguish between direct and indirect chemotactic effects of FasL. Significantly, reduced neutrophil influx into the intestines of DSS-treated *gld/gld* mice has been reported in abstract form by another group (47). Conversely, in a different model of colitis, which was induced by a single local administration of trinitrobenzene sulfonic acid, *gld/gld* mice showed more severe inflammation (48). It is unclear whether differences in the mode of induction of colitis or perhaps in the animal colony produced the discordant results.

The increased tumor number in the gldxMin mice correlates with decreased neutrophil infiltration. This suggests a model whereby neutrophils recruited by FasL in the WT mice act to suppress tumor development. In the *gld* mice where neutrophil recruitment is significantly diminished, more tumors develop unchallenged. The role of inflammatory cells in tumor development is currently a topic of much interest. There is significant data to suggest that inflammatory cells, particularly macrophages and neutrophils, can promote tumor progression through their production of protumorigenic factors, such as vascular endothelial growth factor and cyclooxygenase-2 (38, 49, 50). The majority of the data supporting this concept come from human and animal models of malignant disease. Evidence suggests that factors secreted by the tumor cells are responsible for converting a normal host antitumor response into a process that benefits the tumor (38). In the $Apc^{Min/+}$ model, the tumors are at a relatively early stage and may not yet produce the factors responsible for converting cells to a protumorigenic state. If this is true, we would anticipate that if these tumors did progress to malignancy, then the WT tumors harboring increased numbers of neutrophils may actually become more advanced than the *gld* tumors. In future

work, we will use models of malignant intestinal disease to test this concept as well as whether the Fas counterattack will play a role in such circumstances. Recent data showing that human colon tumors with high numbers of infiltrating leukocytes, particularly T cells, have a significantly improved prognosis (51) make this an important area of investigation.

In conclusion, we have shown that the Fas counterattack is not an important process in a well-characterized spontaneous model of intestinal tumor development. Instead, we find that lack of functional FasL correlates with decreased neutrophil infiltration of tumors and overall higher tumor number. We suggest therefore that, at least in benign intestinal tumors, FasL expression is more relevant as a mediator of neutrophil migration than as a means of overcoming immunosurveillance.

Acknowledgments

Received 12/5/2006; revised 2/26/2007; accepted 3/15/2007.

Grant support: R01 CA60867 (L.M. Matrisian) and pilot project funding from the National Cancer Institute Specialized Program of Research Excellence in Gastrointestinal Cancer grant 5P50 CA095103 (B. Fingleton).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Tanaka M, Itai T, Adachi M, Nagata S. Downregulation of Fas ligand by shedding. Nat Med 1998;4:31–6.
2. Powell WC, Fingleton B, Wilson CL, Boothby M, Matrisian LM. The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. Curr Biol 1999;9:1441–7.
3. Vargo-Gogola T, Crawford HC, Fingleton B, Matrisian LM. Identification of novel matrix metalloproteinase-7 (matrilysin) cleavage sites in murine and human Fas ligand. Arch Biochem Biophys 2002;408:155–61.
4. Ju S-T, Panka DJ, Cul H, et al. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature 1995;373:444–8.
5. French LE, Hahne M, Viard I, et al. Fas and Fas ligand in embryos and adult mice: ligand expression in several immune-privileged tissues and coexpression in adult tissues characterized by apoptotic cell turnover. J Cell Biol 1996;133:335–43.
6. Thorburn A. Death receptor-induced cell killing. Cell Signal 2004;16:139–44.
7. Takahashi T, Tanaka M, Brannan CI, et al. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell 1994;76:969–76.
8. Roths JB, Murphy ED, Eicher EM. A new mutation, gld, that produces lymphoproliferation and autoimmunity in C3H/HeJ mice. J Exp Med 1984;59:1–20.
9. Lynch DH, Watson ML, Alderson MR, et al. The mouse Fas-ligand gene is mutated in gld mice and is part of a TNF family gene cluster. Immunity 1994;1:131–6.
10. O'Connell J, Bennett MW, O'Sullivan GC, Collins JK, Shanahan F. The Fas counterattack: cancer as a site of immune privilege. Immunol Today 1999;20:46–52.
11. Igney FH, Behrens CK, Krammer PH. Tumor counterattack—concept and reality. Eur J Immunol 2000;30:725–31.
12. Hahne M, Rimoldi D, Schroter M, et al. Melanoma cell expression of Fas (Apo1/CD95) ligand: implications for tumor immune escape. Science 1996;274:1363–6.
13. Strand S, Hofmann WJ, Hug H, et al. Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells—a mechanism of immune evasion? Nat Med 1996;2:1361–6.
14. Walker PR, Saas P, Dietrich P-Y. Tumor expression of Fas ligand (CD95L) and the consequences. Curr Opin Immunol 1998;10:564–72.
15. Ottonello L, Tortolina G, Amelotti M, Dallegri F. Soluble Fas ligand is chemotactic for human neutrophilic polymorphonuclear leukocytes. J Immunol 1999;162:360–1.
16. Seino K-I, Iwabuchi K, Kayagaki N, et al. Chemotactic activity of soluble Fas ligand against phagocytes. J Immunol 1998;161:4484–8.
17. Seino K, Kayagaki N, Okumura K, Yagita H. Antitumor effect of locally produced CD95 ligand. Nat Med 1997;3:165–70.
18. Arai H, Gordon D, Nabel EG, Nabel GJ. Gene transfer of Fas ligand induces tumor regression *in vivo*. Proc Natl Acad Sci U S A 1997;94:13862–7.
19. Moser AR, Luongo C, Gould KA, et al. ApcMin: a mouse model for intestinal and mammary tumorigenesis. Eur J Cancer 1995;31A:1061–4.
20. Su LK, Kinzler KW, Vogelstein B, et al. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science 1992;256:668–70.
21. Roberts RB, Min L, Washington MK, et al. Importance of epidermal growth factor receptor signaling in establishment of adenomas and maintenance of carcinomas during intestinal tumorigenesis. Proc Natl Acad Sci U S A 2002;99:1521–6.
22. Kilpatrick L, McCawley L, Nachiappan V, et al. α-1-Antichymotrypsin inhibits the NADPH oxidase-enzyme complex in phorbol ester-stimulated neutrophil membranes. J Immunol 1992;149:3059–65.
23. Suda T, Takahashi T, Golstein P, Nagata S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 1993;75:1169–78.
24. Belluco C, Esposito G, Bertorelle R, et al. Fas ligand is up-regulated during the colorectal adenoma-carcinoma sequence. Eur J Surg Oncol 2002;28:120–5.
25. Strand S, Galle PR. Immune evasion by tumours: involvement of the CD95 (APO-1/Fas) system and its clinical implications. Mol Med Today 1998;4:63–8.
26. Luongo C, Moser AR, Gledhill S, Dove WF. Loss of Apc+ in intestinal adenomas from Min mice. Cancer Res 1994;54:5947–52.
27. Fingleton B, Vargo-Gogola T, Crawford HC, Matrisian LM. Matrilysin expression selects for cells with reduced sensitivity to apoptosis. Neoplasia 2001;3:459–68.
28. Sayers TJ, Brooks AD, Seki N, et al. T cell lysis of murine renal cancer: multiple signaling pathways for cell death via Fas. J Leukoc Biol 2000;68:81–6.
29. Zhang HG, Fleck M, Kern ER, et al. Antigen presenting cells expressing Fas ligand down-modulate chronic inflammatory disease in Fas ligand-deficient mice. J Clin Invest 2000;105:813–21.
30. Okayasu I, Hatekeyama S, Yamada M, et al. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology 1990;98:694–702.
31. O'Connell J, Bennett MW, O'Sullivan GC, et al. Fas ligand expression in primary colon adenocarcinomas: evidence that the Fas counterattack is a prevalent mechanism of immune evasion in human colon cancer. J Pathol 1998;186:240–6.
32. O'Connell J, Bennett MW, O'Sullivan GC, et al. Expression of Fas (CD95/Apo-1) ligand by human breast cancers: significance for tumor immune privilege. Clin Diagn Lab Immunol 1999;6:457–63.
33. O'Connell J, O'Sullivan GC, Collins JK, Shanahan F. The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J Exp Med 1996;184:1075–82.
34. Restifo NP. Not so Fas: re-evaluating the mechanisms of immune privilege and tumor escape. Nat Med 2000;6:493–5.
35. Igney FH, Behrens CK, Krammer PH. CD95L mediates tumor counterattack *in vitro* but induces neutrophil-independent tumor rejection *in vivo*. Int J Cancer 2004;113:78–87.
36. Ryan AE, Shanahan F, O'Connell J, Houston AM. Addressing the "Fas counterattack" controversy: blocking fas ligand expression suppresses tumor immune evasion of colon cancer *in vivo*. Cancer Res 2005;65:9817–23.
37. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity 2004;21:137–48.
38. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 2004;4:71–8.
39. Aoki K, Kurooka M, Chen J-J, et al. Extracellular matrix interacts with soluble CD95L: retention and enhancement of cytotoxicity. Nat Immunol 2001;2:333–7.
40. Chen J-J, Sun Y, Nabel GJ. Regulation for the proinflammatory effects of Fas ligand (CD95L). Science 1998;282:1714–7.
41. Hohlbaum AM, Gregory MS, Ju ST, Marshak-Rothstein A. Fas ligand engagement of resident peritoneal macrophages *in vivo* induces apoptosis and the production of neutrophil chemotactic factors. J Immunol 2001;167:6217–24.
42. Hohlbaum AM, Moe S, Marshak-Rothstein A. Opposing effects of transmembrane and soluble Fas ligand expression in inflammation and tumor cell survival. J Exp Med 2000;191:1209–19.
43. Seino K, Ogino T, Fukunaga K, et al. Attempts to reveal the mechanism of CD95-ligand-mediated inflammation. Transplant Proc 1999;31:1942–3.
44. Wilson CL, Heppner KJ, Labosky PA, Hogan BL, Matrisian LM. Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc Natl Acad Sci U S A 1997;94:1402–7.
45. Fingleton B, Powell WC, Crawford HC, Couchman JR, Matrisian LM. A rat monoclonal antibody that recognizes pro- and active MMP-7 indicates polarized expression *in vivo*. Hybridoma 2007;26:22–7.
46. Kang S-M, Braat D, Schneider DB, et al. A non-cleavable mutant version of Fas ligand does not prevent neutrophilic destruction of islet transplants. Transplantation 2000;69:1813–7.
47. Beck PL, Wong J, and Muruve D. The regulation of Fas and Fas ligand alters the severity of a murine model of colitis. Gastroenterology 2002;122:A269–270.
48. Sayani FA, Keenan CM, Van Sickle MD, et al. The expression and role of Fas ligand in intestinal inflammation. Neurogastroenterol Motil 2004;16:61–74.
49. Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420:860–7.
50. Josephy PD, Coomber BL. The 1996 Veylien Henderson Award of the Society of Toxicology of Canada. Current concepts: neutrophils and the activation of carcinogens in the breast and other organs. Can J Physiol Pharmacol 1998;76:693–700.
51. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313:1960–4.

Loss of Functional Fas Ligand Enhances Intestinal Tumorigenesis in the Min Mouse Model

Barbara Fingleton, Kathy J. Carter and Lynn M. Matrisian

Cancer Res 2007;67:4800-4806.