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Ovarian Carcinoma Expresses the NKG2D Ligand Letal and Promotes the Survival and Expansion of CD28$^-$ Antitumor T Cells

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ABSTRACT

The role of the NKG2D immunoreceptor and its ligands in antitumor immune response is incompletely understood. Here, we report that effector immune cells infiltrating ovarian carcinoma are mostly CD8$^+$ lymphocytes lacking CD28 but expressing the NKG2D costimulatory receptor. Human ovarian carcinoma expresses the novel NKG2D ligand lymphocyte effector cell toxicity-activating ligand (Letal). Letal was found to be an independent prognosticator of improved survival in advanced ovarian cancer. Higher levels of tumor-derived Letal were associated with stronger lymphocyte infiltration. Letal exerted marked costimulatory effects and induced type-1 polarization in CD8$^+$CD28$^-$ tumor-infiltrating lymphocytes *ex vivo*. Letal engagement increased the expression of the glucose transporter Glut-1, enhanced glucose up-take, and protected CD8$^+$ lymphocytes from cisplatin-induced killing. Letal also down-regulated the expression of Fas in CD8$^+$ cells and rendered them resistant to Fas ligand-induced apoptosis. Our results indicate that Letal promotes tumor immune surveillance by promoting the survival and intratumoral expansion of antitumor cytotoxic lymphocytes. We propose that Letal could be used for the *ex vivo* expansion of apoptosis-resistant tumor-reactive cytotoxic lymphocytes for adoptive transfer.

INTRODUCTION

Induction of tumor-specific CTLs recognizing MHC-I-restricted tumor antigens and the activation of natural killer (NK) cells play a critical role in antitumor immune response (1–3). The balance between activating and inhibitory ligands expressed in tumors may critically affect the function of effector lymphocytes and the efficacy of antitumor immune response. Tumor-associated Fas ligand (FasL) and other tumor necrosis factor-related death ligands may induce apoptotic depletion of tumor-infiltrating CTLs (4, 5). However, the adoptive transfer of *ex vivo* expanded tumor-infiltrating lymphocytes (TILs) has led to successful engraftment and regression of metastatic tumors (6). Furthermore, tumors known to express FasL such as ovarian cancer (7) may exhibit abundant TILs, which are associated with dramatically improved outcome, as we recently reported (3). Thus, tumor-specific T-cell populations may persist and expand in tumors despite tumor-derived death signals.

The NKG2D immunoreceptor is expressed on most CD8$^+$ T cells, $\gamma\delta$ T cells, and NK cells and serves as one of the most potent activating receptors for effector lymphocytes in peripheral tissues (8, 9). There is evidence that ligands for NKG2D mark tumor cells for immune cell-mediated killing. Ectopic expression of NKG2D ligands has resulted in immune-mediated rejection of tumors in mouse models (10, 11). Furthermore, skin-associated NKG2D$^+$ $\gamma\delta$ T cells use a mechanism dependent on NKG2D engagement to mediate destruction of carcinoma cells (12). However, little is known about the role of NKG2D ligands in human tumors.

We have recently reported the identification and functional characterization of a novel MHC-I-related ligand for the NKG2D receptor, which we named lymphocyte effector cell toxicity-activating ligand (Letal; Ref. 13). Letal provides a costimulatory signal to CD8$^+$ lymphocytes, inducing their proliferation and activation. On NK cells, Letal directly delivers a primary stimulatory signal, which triggers the killing of chemoresistant cancer cells. Here, we report that effector lymphocytes infiltrating ovarian carcinoma are mostly CD8$^+$ cells lacking CD28 but expressing the NKG2D costimulatory receptor. Letal is up-regulated in advanced ovarian carcinomas, which despite FasL expression, exhibit intratumoral T-cell accumulation. Letal expression correlated with lymphocyte infiltration in tumors, whereas Letal was an independent prognostic factor predicting improved survival. Letal exerted marked costimulatory effects on T-cell receptor-mediated proliferation of CD8$^+$CD28$^-$ lymphocytes. Letal engagement increased the expression of the glucose transporter Glut-1 and enhanced glucose up-take in CD8$^+$ lymphocytes. Letal also protected cytotoxic lymphocytes from cisplatin-induced killing and down-regulated the expression of Fas in CD8$^+$ cells, thus decreasing Fas-dependent apoptosis. These results suggest an important role for Letal in the homeostasis of peripheral CD8$^+$ effector T cells and the immune defense against tumors in the human and offer new therapeutic opportunities in the field of adoptive lymphocyte therapy of cancers.

MATERIALS AND METHODS

Tissues and Cells. Normal human ovaries were obtained from the Cooperative Human Tissue Network. Frozen ovarian tumor specimens were obtained from the University of Turin (Turin, Italy), as described previously (3). These comprised 2 normal postmenopausal ovaries, 4 benign cystadenomas, 4 low malignant potential tumors, 9 Federation of Gynecologists and Obstetricians stage I, and 43 stage III ovarian carcinomas. Twenty-one percent of stage III tumors analyzed here had not been included in an earlier study (3). Cancer specimens comprised samples from the primary tumor site (ovarian mass) and were collected at the time of initial exploratory surgery from patients previously untreated with chemotherapy. Optimal surgical debulking was defined as residual individual tumor nodules measuring $\leq$1 cm in diameter. Chemotherapy comprised platinum, platinum-cyclophosphamide, or platinum-paclitaxel. Complete response to therapy was defined by normalization of physical examination, abdomino-pelvic computed tomography scan and serum CA-125. Noncomplete response included partial response ($\geq$50% decrease in the sum of greater tumor dimensions by computed tomography) or no response ($<$50% decrease or any increase in tumor). The duration of overall survival was the interval between diagnosis and death; data were censored at the last follow-up

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Note: J. R. Conejo-Garcia and F. Benencia contributed equally to this work.

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date for patients who were alive at the time of analysis. Histopathological classification was independently confirmed at the University of Pennsylvania. Institutional approval was received from the University of Turin for the procurement of the specimens and by the University of Pennsylvania for the transfer and analysis of the specimens, as described elsewhere (3).

Fresh ovarian cancer specimens were obtained from the University of Pennsylvania following written informed consent and were immediately minced, digested with collagenase A (Roche, Mannheim, Germany), and subjected to Ficol gradient centrifugation to derive single cell suspensions. $CD8^+$ lymphocytes were sorted from freshly digested tumors or fresh ascites on a MoFlo cell sorter (Cytomation, Fort Collins, CO) using an anti-CD8 antibody (PharMingen, Torrey Pines, CA). Tumor-infiltrating or tumor-associated $CD8^+CD28^-$ lymphocytes were sorted using a combination of anti-CD8 monoclonal antibody (mAb; PharMingen) and anti-CD-28 mAb (9.3, kindly provided by Dr. Carl H. June University of Pennsylvania). Fresh peripheral blood lymphocytes were obtained by leukapheresis and elutriation as described elsewhere (14). $CD8^+$ cells were procured by negative selection using the OKT4 antibody (Ortho-Clinical Diagnostics, Raritan, NJ) to separate $CD4^+$ cells from the elutriate, as described elsewhere (14). Institutional approval was received from the University of Pennsylvania for the procurement of specimens and the performed analyses, according to The Health Insurance Portability and Accountability Act regulations. The K32 cell line, a K562 erythroleukemia cell line stably expressing the human low-affinity Fcγ receptor CD32 (14), was a generous gift by Dr. Carl H. June (from University of Pennsylvania).

Letal Constructs and Generation of Anti-Letal Antibody. MIGR1 retrovirus encoding enhanced green fluorescent protein (GFP) was generously provided by Dr. Warren Pear (University of Pennsylvania). Letal (GenBank accession no. AY069961) was cloned from the ovarian carcinoma cell line A2008, as previously described (13). The entire Letal open reading frame was inserted in the MIGR1 retroviral vector upstream of GFP, preceded by an internal ribosome entry site (IRES), as previously described (13). K32 cells were transfected with $Letal^+GFP^+$ or with control GFP^+ MIGR1. $Letal^+$ or mock transductants expressing equivalent levels of GFP were sorted and cultured by standard procedures.

To generate a polyclonal anti-Letal antibody (Ab), healthy 6-week-old female C57BL/6 mice were immunized with 25 μg of Letal cDNA cloned in the pcDNA 3.1 expression vector (Invitrogen, Carlsbad, CA), which was injected i.p. weekly for 3 weeks. Sera were collected by bleeding mice 7 and 14 days after the last vaccination. Sera were screened for anti-Letal Ab by flow cytometry, using cell lines transfected with the complete Letal open reading frame.

Real-Time Quantitative Reverse Transcription-PCR. Letal expression was analyzed by TaqMan PCR analysis as described previously (13, 15). The Letal system consisted of the following primers: Letal.F, 5′-CTCAGGATGCTCCTTTGTGACAT-3′, Letal.R, 5′-CTTCACGTTGACAAAACATCTCG-3′; and the probe Letal.P, 5′-(FAM)CCCAGATAAAGACCAGTGATCCTTCCACT(TAMRA)-3′. We normalized the cDNA load to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with primers GAPDH F: 5′-CCTGCACCACCAACTGCTTA-3′ and GAPDH R: 5′-CATGAGTCCTTCCACGATACCA-3′ and the probe GAPDH.P: 5′(FAM)CCTGGCCAAGGTCATCCATGACAAC(TAMRA)-3′. Letal was considered to be present in tumor specimens if more than five copies of Letal mRNA were detected for every 10^6 copies of GAPDH mRNA. Twenty-eight stage III tumors were classified as Letal positive, whereas the remaining 15 stage III tumors were considered Letal negative.

Apoptosis Assay. Apoptosis was induced *in vitro* by incubating lymphocytes in 50 μM cisplatin (Sigma, St. Louis, MO) for 17 h or by exposing them to agonistic mAb EOS9.1 against Fas/CD95 (PharMingen; 0.1 μg/ml) for 18 h. The percentage of apoptotic cells was determined by flow cytometry using the annexin-V detection kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Fas/CD95 expression was determined by flow cytometry by using EOS9.1 as a primary Ab and a phycoerythrin-labeled antimouse IgM (R6-60.2; PharMingen) as secondary Ab.

Apoptotic T cells in tumors were visualized by performing fluorescent *in situ* terminal deoxynucleotidyl transferase-mediated nick end labeling assay combined with CD3 immunofluorescence. Terminal deoxynucleotidyl transferase-mediated nick end labeling was carried out first using the ApopTag *in situ* detection kit (Intergen, Purchase, NY) according to the manufacturer's instructions. Briefly, tumor tissue sections were fixed with 1% paraformaldehyde in PBS, followed by cold ethanol and acetic acid postfixation. After incubation with residues of digoxigenin nucleotide and terminal deoxynucleotide transferase for 1 h at 37°C, sections were incubated with FITC-labeled anti-digoxigenin antibody. CD3 immunofluorescence was then performed with a rabbit anti-CD3 (Dako, Carpinteria, CA) as previously described, using rhodamine-conjugated secondary Ab (3).

$CD8^+$ Lymphocyte Stimulation and Cytokine Assay. To analyze the effects of Letal on T-cell proliferation and cytokine production *in vitro*, we used a previously described artificial antigen-presenting cell system based on K32 cells stably expressing the human low-affinity Fcγ receptor CD32 (14). K32 cells were additionally transduced with $Letal^+$ or with empty vector as described above, irradiated with lethal gamma rays (100 Gy), and washed twice with RPMI medium. $Letal^+$ K32 cells or mock transductants were loaded, when indicated, with anti-CD3 (OKT3; Ortho-Clinical Diagnostics) or anti-CD3 plus anti-CD28 (9.3, kindly provided by Dr. Carl H. June, University of Pennsylvania) mAbs at 0.5 μg/ml for 10 min at room temperature. Loaded artificial antigen-presenting cells were mixed with $CD8^+$ T cells at a 1:2 ratio, and the T-cell concentration was maintained at 0.5×10^6 cells/ml throughout the culture. Cultures were pulsed with 1 μCi of [^{3}H]thymidine from day 2 to day 5, and incorporated radioactivity was determined using a 1450 Microbeta scintillation counter (Wallac, Turku, Finland). We determined the amounts of secreted IFN-γ by commercial ELISA, following the manufacturer's instructions (R&D Systems). Flow cytometry was performed with a FACScalibur (BD Biosciences, San Jose, CA).

Glut-1 Expression and Glucose Metabolism Analysis. Glut-1 expression was quantified by flow cytometry through intracellular staining with rabbit anti-Glut-1 Ab (Research Diagnostics, Flanders, NJ) as described previously (16). Glucose uptake was measured as described previously (16). Briefly, stimulated T cells were incubated for 15 min at 37°C in glucose uptake buffer [8.1 mM Na_2HPO_4, 1.4 mM KH_2PO4, 2.6 mM KCl, 136 mM NaCl, 0.5 mM $MgCl_2$, and 0.9 mM $CaCl_2$ (pH 7.4)] to deplete intracellular glucose stores. Triplicate samples of 1×10^6 cells were incubated with 1 μCi of [^{3}H]2-deoxyglucose (NEN, Boston, MA) in glucose uptake buffer for 2 min at room temperature and immediately spun through a layer of bromododecane (Sigma) into 20% perchloric acid/8% sucrose, stopping the reaction, and separating the cells from unincorporated [^{3}H]2-deoxyglucose. The perchloric acid/sucrose/T-cell layer was removed and analyzed by liquid scintillation as above.

Laser Capture Microdissection and Immunohistochemistry. Tumor islets were microdissected en bloc from 6-μm thick hematoxylin-stained cryosections of ovarian cancer specimens using the μCUT Laser-MicroBeam System (SL Microtest, Jena, Germany), according to the manufacturer's instructions. Total RNA was immediately extracted from microdissected tissue using the PicoPure RNA Isolation kit (Arcturus, Mountain View, CA). Immunohistochemistry was performed exactly as previously described (3), using anti-CD8 mAb (SK-1), anti-CD57 mAb (NK-1, both from PharMingen), anti-CD45 mAb (X16/99; Novocastra, Newcastle, United Kingdom), rabbit anti-CD3 Ab (Dako), anti-NKG2D Ab (149810; R&D Systems), and mouse anti-Letal serum generated in our laboratory as described above. Mouse preimmune serum was used as a negative control for the anti-Letal serum. Horse serum (1/10 dilution) was used as a negative control for the secondary Abs. $CD3^+$ T cells were counted manually in 15–20 high-power fields. Tumors were considered to be positive for intratumoral T cells if greater than five $CD3^+$ cells were detected/high power field in >10 high-power fields.

Statistical Analysis. A χ^2 test was used to evaluate differences in clinicopathological variables between patients whose tumors were positive or negative for Letal or NKG2D in tumor islets. Kaplan-Meier curves were used to estimate survival and were compared with the use of long-rank statistics. Univariate and multivariate Cox proportional hazard models were used to estimate the unadjusted and adjusted hazard ratios, respectively. Individual survival curves were plotted for eight groups of patients differing in three significant predictive factors: surgical debulking (optimal *versus* suboptimal); intratumoral T cells (tumors positive *versus* negative); and Letal expression (present *versus* absent in tumor islets). After comparisons of individual survival curves of these eight groups of patients, a final model was obtained by combining those groups whose survival curves were not significantly different. Descriptive statistical analyses were performed with SPSS software; survival analyses were performed with SAS software.

RESULTS

$CD8^+$ T Cells Represent the Predominant $NKG2D^+$ Lymphocytic Population-Infiltrating Advanced Ovarian Carcinomas. We first evaluated the presence of total leukocytes in 100 snap-frozen specimens of ovarian carcinomas by immunohistochemistry. $CD45^+$ leukocytes were detected in different proportions within tumor cell islets, in surrounding stroma, or both. Surprisingly, $CD45^+$ cells represented up to 45% of total cells in many specimens (Fig. 1*A*). Next, we examined the expression of NKG2D in these tumors. More than 50% of tumor islets were infiltrated by $NKG2D^+$ cells, which, in average, represented 15% of the total leukocytes in stage III tumors (Fig. 1*B*). Interestingly, seven tumor specimens exhibited diffuse NKG2D staining in tumor islets in the absence of TILs, suggesting that the expression of NKG2D may not be restricted to lymphocytes in human tumors (data not shown).

We investigated the relative contribution of cytotoxic effector immune cells in these tumors. The vast majority of them were found to be $CD8^+$ T cells (Fig. 1*C*). In contrast, $CD57^+$ NK cells were scarcely represented in most specimens analyzed (Fig. 1*D*), suggesting a predominant role of T-cell-mediated responses in immune surveillance against established tumors. Lower stage tumors contained significantly fewer infiltrating lymphocytes than advanced ovarian carcinomas (data not shown), indicating that a certain degree of invasion and dedifferentiation is necessary to trigger a sustained immune response. To assess the viability of intratumoral T cells in these tumors, we performed fluorescent *in situ* terminal deoxynucleotidyl transferase-mediated nick end labeling combined with CD3 immunofluorescence. DNA fragmentation (apoptosis) was seen in $<10\%$ of intratumoral T cells (Fig. 1*E*), indicating that the majority of intratumoral T cells are viable.

$CD8^+$ T-Cell Infiltration Is Associated with Letal Overexpression in Human Ovarian Carcinoma. We next analyzed Letal expression in ovarian cancer at the protein level. Strong Letal-specific immunostaining was detected in $>50\%$ of stage III carcinomas. Importantly, Letal expression was localized mainly to tumor cells (Fig. 1*F*), attesting the specificity of immunostaining. These results are in agreement with our previous study that select ovarian cancer cell lines constitutively express Letal mRNA (13). We also observed a positive signal in select tumor-infiltrating leukocytes within the stroma surrounding tumor islets (data not shown).

Because ovarian cancer progression was found to be associated with increasing frequency of infiltrating lymphocytes, we quantified Letal mRNA during tumor progression in 62 ovarian neoplasms and control postmenopausal ovaries. Using real-time quantitative PCR, we found that expression of Letal mRNA was low in normal ovaries and in benign or low malignant potential tumors, whereas stage I to III ovarian carcinomas exhibited 40-fold higher expression ($P < 0.01$; Fig. 2*A*).

We have previously demonstrated that T cells infiltrating tumor islets (intratumoral T cells) are detected in ~55% of ovarian cancers, whereas T cells are detectable only in the stroma surrounding tumor islets in the remainder tumors (3). We have also demonstrated that

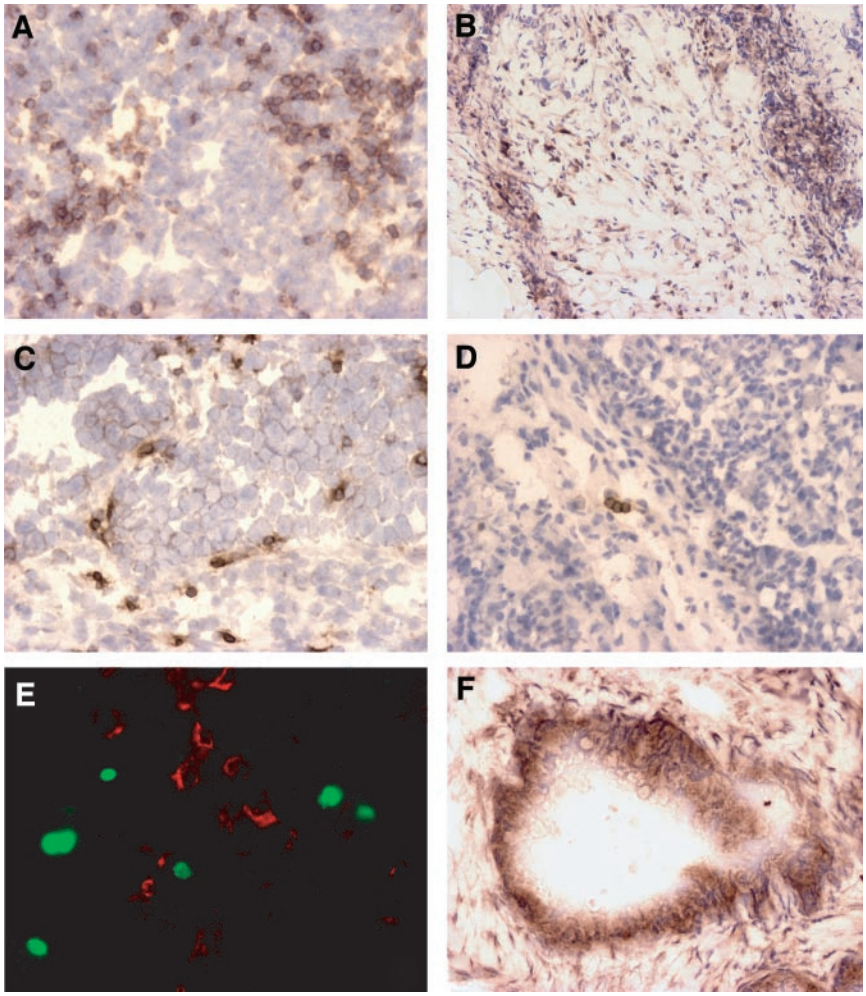

Fig. 1. Immunohistochemical staining of advanced human ovarian carcinomas. *A*, a high frequency of $CD45^+$ leukocytes is seen after staining with anti-CD45 mAb (magnification, ×20); *B*, a high proportion of $NKG2D^+$ cells is seen. In average, these represent 15% of total leukocytes (magnification, ×10); *C*, $CD8^+$ cells are noted within a tumor islet (magnification, ×20). *D*, $CD57^+$ NK cells are only occasionally present in advanced ovarian carcinoma ($<1\%$ of total $CD45^+$ cells); *E*, double fluorescent terminal deoxynucleotidyl transferase-mediated nick end labeling/anti-CD3 immunostaining demonstrates absence of apoptosis, as detected by terminal deoxynucleotidyl transferase-mediated nick end labeling (FITC, *green*), in $CD3^+$ tumor-infiltrating lymphocytes (rhodamine, *red*). *F*, Letal protein is expressed predominantly by tumor cells in ovarian carcinomas. Nuclei were counterstained with hematoxylin. These images are representative of stage III ovarian carcinomas with intratumoral T cells.

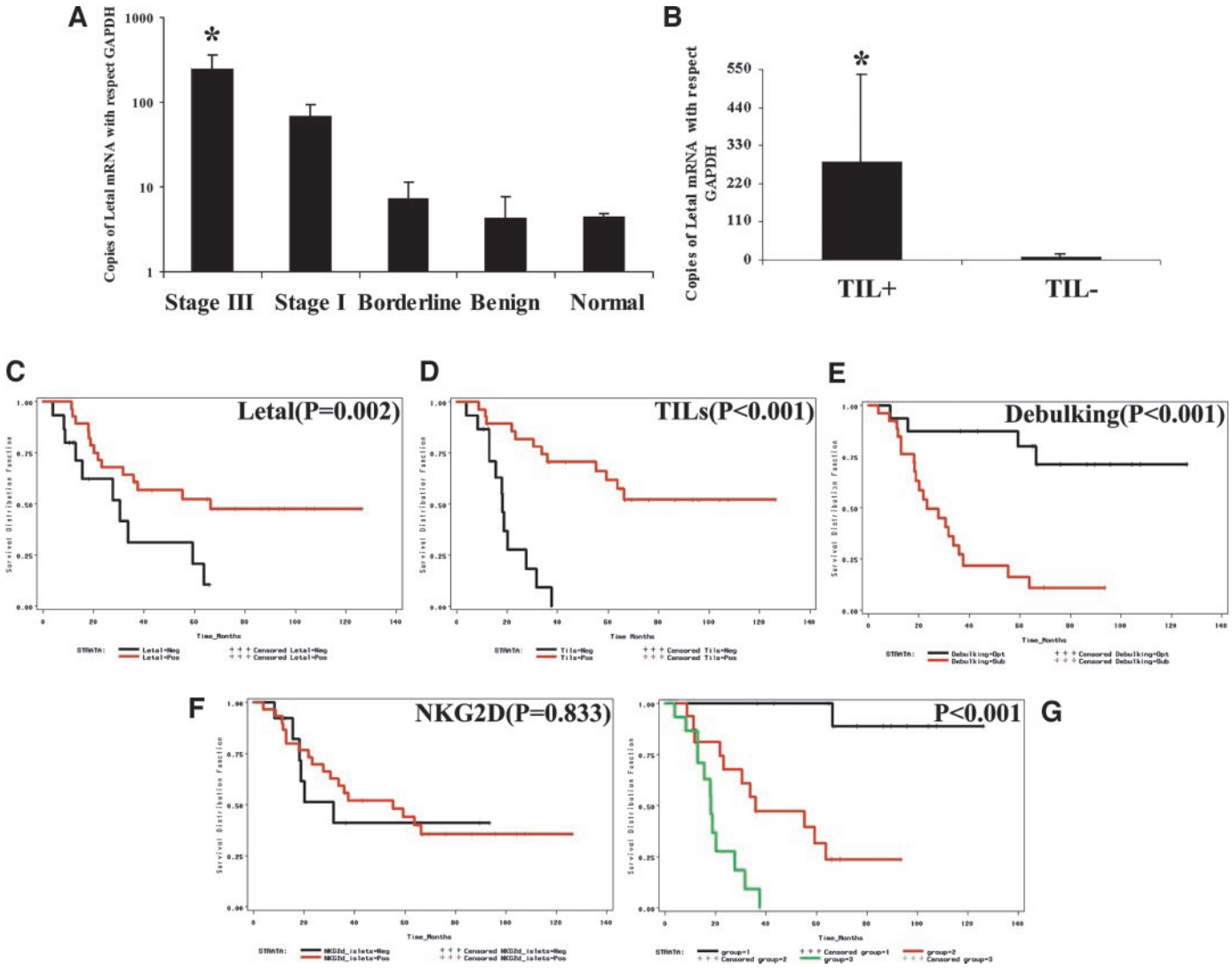

Fig. 2. Letal expression increases with tumor progression but is associated with better outcome. *A*, quantification of Letal mRNA levels by TaqMan PCR in human normal ovaries, benign tumors ($n = 6$), borderline tumors ($n = 4$), stage I ($n = 9$), and stage III ovarian carcinomas ($n = 43$). *B*, Letal mRNA expression analyzed by TaqMan PCR in tumor islets harboring intratumoral T cells and tumor islets lacking intratumoral T cells, isolated by laser capture microdissection. *C–E*, Kaplan-Meier curves for overall survival based on the presence (red) *versus* absence (*black*) of Letal mRNA (*C*), the presence (*red*) *versus* absence (*black*) of intratumoral T cells (*D*), suboptimal (*red*) *versus* optimal (*black*) debulking (*E*), and the presence (*red*) *versus* absence (*black*) of NKG2D staining in tumor islets (*F*). *G*, Kaplan-Meier curves for overall survival in patients with tumors characterized by optimal debulking plus tumor-infiltrating lymphocytes (TILs) present plus Letal present (*black*), TILs present *plus* either optimal debulking or Letal present (*red*), and TILs present plus any combination of debulking and Letal mRNA expression (*green*).

simultaneous stimulation of the T-cell receptor (TCR), and Letal induces proliferation of CTLs *in vitro* (13). To investigate the role of Letal in the expansion of TILs *in vivo*, we produced highly pure samples of tumor islets from tumors with or without intratumoral T cells by laser capture microdissection. Real-time PCR of 19 stage III specimens revealed 30-fold higher Letal mRNA expression in islets infiltrated by T cells ($n = 12$ tumors) compared with islets lacking T cells ($n = 9$ tumors; $P = 0.041$; Fig. 2*B*). Thus, increased Letal expression is detected in tumors with intratumoral T cells and increased T-cell infiltration. Because most ovarian tumors express MHC-I by immunohistochemistry (17) and flow cytometry (our unpublished observations), these data suggest that Letal is involved in the enrichment of T cells in tumor islets.

We examined the association of Letal expression (at the mRNA level) in tumor islets with the presence of intratumoral T cells, the expression of NKG2D, and known clinicopathological prognosticators, including tumor histotype, the degree of tumor debulking, the inclusion of paclitaxel in the chemotherapy regimen, and the response to chemotherapy (Table 1). Letal was associated with the presence of intratumoral T cells ($P = 0.06$) but not with the presence of NKG2D in tumor islets. No association was found between Letal and histological type of tumors (serous, mucinous, or endometrioid *versus* clear-cell or undifferentiated), surgical debulking (optimal *versus* suboptimal), inclusion of paclitaxel in the chemotherapy regimen (included *versus* not included), or response to chemotherapy (complete *versus* noncomplete; Table 1). An association was seen between NKG2D expression in tumor islets (at the protein level) and intratumoral T cells ($P < 0.01$). No association was found between the expression of NKG2D and tumor histotype, surgical debulking, inclusion of paclitaxel in the chemotherapy regimen, or response to chemotherapy (Table 1).

Expression of Letal Is an Independent Prognostic Factor in Ovarian Carcinoma. We have previously demonstrated that patients with ovarian cancer whose tumors exhibit intratumoral T cells experience prolonged survival (3). Univariate analysis revealed significant differences in the survival distributions for patients with Letal-positive and -negative stage III tumors (log-rank test; $P = 0.002$; Fig. 2*C*). Patients whose tumors expressed Letal mRNA had a median duration

Table 1 *Association with other clinicopathological variables*

A. Letal

	Letal present (%)	Letal absent (%)	*P*
Intratumoral T cells			
Present	46.7	75.0	0.063
Absent	53.3	25.0	
NKG2D[a]			
Present	60.0	75.0	0.307
Absent	40.0	25.0	
Debulking			
Optimal	26.7	42.9	0.295
Suboptimal	73.3	57.1	
Histotype			
Serous, mucinous, or endometroid	53.3	71.4	0.235
Clear cell or undifferentiated	46.7	28.6	
Paclitaxel			
Included	40.0	60.7	0.194
Not included	60.0	39.3	
Response to chemotherapy			
Complete	73.3	78.6	0.698
Noncomplete[b]	26.7	21.4	

B. NKG2D

	NKG2D present[a] (%)	NKG2D absent[a] (%)	*P*
Intratumoral T cells			
Present	38.5	76.7	0.016
Absent	61.5	23.3	
Debulking			
Optimal	30.8	40.0	0.565
Suboptimal	69.2	60.0	
Histotype			
Serous, mucinous, or endometroid	46.2	73.3	0.086
Clear cell or undifferentiated	53.8	26.7	
Paclitaxel			
Included	61.5	50.0	0.486
Not included	38.5	50.0	
Response to chemotherapy			
Complete	69.2	80.0	0.443
Noncomplete[b]	30.8	20.0	

[a] In tumor islets.
[b] Comprises partial response and no response.

of overall survival of 66.4 months ($n = 28$; risk ratio = 0.38), as compared with 30.5 months among patients with Letal-negative tumors ($n = 15$). The 5-year overall survival rate was 52% among patients whose tumors expressed Letal mRNA, but only 21% among patients whose tumors were Letal-negative. Thus, although Letal expression increases in advanced stage, it appears to play a protective role in ovarian carcinoma. As expected, the presence of intratumoral T cells (risk ratio = 0.13; $P < 0.01$) and optimal tumor debulking (risk ratio = 0.14; $P < 0.01$) was also associated with prolonged survival (Fig. 2, *D* and *E*). Given than 21% of the patients evaluated in this study were not included in our previous work (3), these data confirm the strength of our previous conclusions. No effect on survival was detected for tumor histotype ($P = 0.367$), the inclusion of paclitaxel in the chemotherapy regimen ($P = 0.368$), or the presence of NKG2D signal in tumor islets ($P = 0.834$; Fig. 2*F*).

In multivariate analysis, expression of Letal mRNA, optimal debulking, and the presence of intratumoral T cells (but not the expression of NKG2D, tumor histotype, or the inclusion of paclitaxel in the chemotherapy regimen) were found to be independent prognosticators of prolonged overall survival in stage III ovarian cancer (Table 2). Individually, the above three significant binary variables resulted in eight classes of patients with various combinations of surgical debulking (optimal *versus* suboptimal), intratumoral T cells (present *versus* absent), and Letal expression (present *versus* absent in tumor islets). On the basis of similar survival curves among select classes, the variables were then grouped to produce three groups of patients, which exhibited significantly different survival curves ($P < 0.01$): (*a*) good prognosis tumors ($n = 12$)—optimal debulking *plus* intratumoral T cells present *plus* Letal present in tumor islets; (*b*) moderate prognosis tumors ($n = 17$)—intratumoral T cells present *plus* either Letal absent or suboptimal debulking or both; and (*c*) poor prognosis tumors ($n = 14$)—intratumoral T cells absent *plus* either suboptimal debulking or Letal absent or both (Fig. 2*G*).

Tumor-Infiltrating $CD8^+$ Lymphocytes Do Not Express CD28 but Can Be Expanded through CD3/Letal Engagement. A significant proportion of peripheral effector $CD8^+$ cells are known to be negative for the costimulatory molecule CD28 and therefore to rely on alternate costimulatory molecules (18). We analyzed the expression of CD28 in tumor-infiltrating or tumor-associated $CD8^+$ lymphocytes freshly procured from three dissociated ovarian tumors and two tumor ascites specimens. Diminished or completely absent expression of CD28 was observed on >80% of tumor-infiltrating CTLs and almost all tumor-associated $CD8^+$ lymphocytes derived from ascites (Fig. 3*A*). Above, we showed that tumor-infiltrating leukocytes express NKG2D by immunostaining (Fig. 1*B*). Hence, we investigated whether Letal engagement could provide costimulation to $CD8^+CD28^-$ lymphocytes sorted from the same specimens. As shown in Fig. 3*B*, CD3/Letal costimulation delivered through $Letal^+$ K32 cells bearing anti-CD3 mAb induced a sustained proliferation of $CD8^+$ cells from all specimens for at least 3 weeks *ex vivo*. Additionally, secretion of IFN-γ in CD3/Letal costimulated tumor-derived CTLs was dramatically increased compared with CTLs activated by CD3 engagement alone (Fig. 3*C*). Thus, Letal provides an important tumor-associated costimulatory molecule recognized by tumor-infiltrating $CD28^-$ CTLs. The above findings collectively support an important role of Letal in intratumoral expansion of TILs in ovarian carcinoma.

Letal Signaling Increases Glucose Transporter Expression and Glucose Uptake during T-Cell Activation. It has been recently reported that lymphocyte activation through CD28 costimulation increases glucose uptake and glycolysis (16). To test whether Letal may have a similar effect during T-cell activation, we stimulated peripheral $CD8^+$ T cells for 20 h with irradiated K32 cells bearing anti-CD3 mAb or anti-CD3/anti-CD28 mAbs or $Letal^+$ K32 cells bearing anti-CD3 mAb or no Ab and analyzed the expression of the glucose transporter Glut-1 by flow cytometry. Activation by cross-linking the TCR/CD3 complex altered Glut-1 expression only in 11% of the cells (Fig. 4*A*). In contrast, stimulation with anti-CD3/Letal or Letal alone led to a marked induction of Glut-1 expression, which was similar to that induced by CD3/CD28 costimulation.

We next measured glucose uptake rates using [^{3}H]2-deoxyglucose in cells stimulated as described above. As expected, CD3/CD28 costimulation increased glucose uptake to previously reported levels, whereas CD3 alone had little effect. Interestingly, a more pronounced increase in glucose uptake was seen in cells stimulated with CD3/Letal or Letal alone compared with CD3/CD28-costimulated cells (Fig. 4*B*). Thus, Letal engagement, alone or in association with TCR engagement, induces signals that prepare lymphocytes for the increased metabolic demands associated with effector functions.

Our studies in ovarian cancer indicated that the presence of intra-

Table 2 *Risk ratios from multivariate Cox proportional hazards model analysis*

Group	Risk ratio	*P*
Good prognosis		
Intratumoral T cells present plus optimal debulking plus Letal present	0.01	<0.001
Moderate prognosis		
Intratumoral T cells present plus either Letal absent or suboptimal debulking or both	0.25	0.005
Poor prognosis		
Intratumoral T cells absent plus either Letal absent or suboptimal debulking or both	1.00	

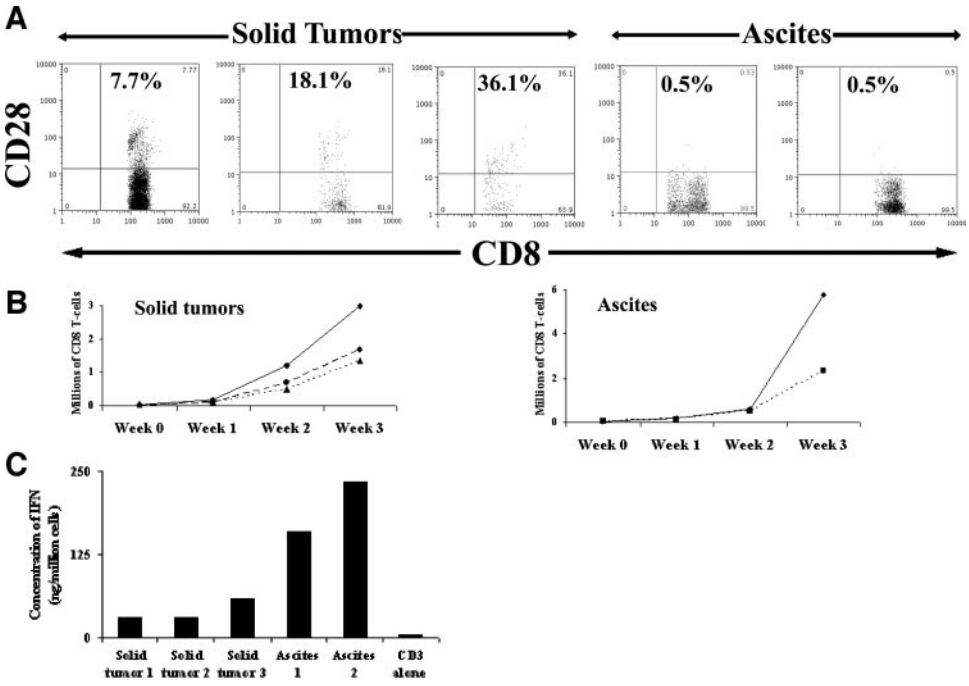

Fig. 3. Letal induces sustained expansion of tumor-infiltrating or tumor-associated $CD28^-$ effector lymphocytes. *A*, most $CD8^+$ lymphocytes in solid tumors and ascites do not express the costimulatory molecule CD28. *B*, sustained expansion of sorted tumor-infiltrating or tumor-associated $CD8^+CD28^-$ lymphocytes through CD3/Letal costimulation provided by $Letal^+$ K32 cells bearing anti-CD3 monoclonal antibody. *Left*, tumor-infiltrating lymphocytes procured from solid tumors; *right*, tumor-associated lymphocytes sorted from tumor ascites. *C*, combined stimulation with anti-CD3 and Letal induces a marked increase in IFN-γ secretion by $CD8^+CD28^-$ cells. Results are compared with a pool of supernatants from the same cells activated with K32 cells bearing anti-CD3 monoclonal antibody alone.

tumoral T cells strongly predicts long remission after cytotoxic chemotherapy (3). However, cytotoxic chemotherapy can deplete tumor-specific effector T cells (19). Because the glycolytic pathway is implicated in lymphocyte survival (20), we investigated whether Letal could protect T cells from genotoxic drug-induced apoptosis. $CD8^+$ lymphocytes from peripheral blood were incubated for 3 days with control media, irradiated K32 cells bearing anti-CD3 mAb or anti-CD3/anti-CD28 mAbs, or $Letal^+$ K32 cells bearing anti-CD3/Letal or no Ab and were subsequently exposed to 50 μM cisplatin for 17 h. A higher percentage of apoptotic cells was detected among $CD8^+$ lymphocytes prestimulated with anti-CD3 than among resting $CD8^+$ lymphocytes. In contrast, markedly lower prevalence of apoptosis was observed among $CD8^+$ lymphocytes costimulated with CD3/Letal or CD3/CD28 (Fig. 4*C*). Importantly, $CD8^+$ cells prestimulated with Letal alone exhibited markedly stronger resistance to platinum-induced apoptosis. Collectively, the above data demonstrate that Letal engagement protects $CD8^+$ T cells from apoptosis induced by genotoxic drugs.

Letal Engagement Protects $CD8^+$ T Cells from FasL-Induced Apoptosis. We showed above that intratumoral T cells show low prevalence of apoptosis *in situ*. We have also reported that intratumoral T cells exhibit evidence of activation and proliferation in ovarian carcinomas (3). These data are potentially in contrast with evidence that ovarian carcinoma cells express *ex vivo* FasL and can induce Fas-mediated apoptosis in cultured TILs (7). We sought to confirm the prevalence of FasL expression *in vivo* in 42 stage III ovarian carcinoma specimens. Strong FasL immunoreactivity was detected in tumor islets (Fig. 5*A*) and/or in stromal leukocytes in select specimens. We surmised that Letal renders activated T cells resistant to tumor necrosis factor-related apoptotic signals. Peripheral $CD8^+$ T cells were incubated for 4 days with control media, irradiated K32 cells bearing anti-CD3 mAb or anti-CD3/anti-CD28 mAbs, or $Letal^+$ K32 cells bearing anti-CD3 mAb, and expression of Fas/CD95 was measured by flow cytometry. Fas expression in unstimulated lymphocytes progressively increased over 4 days of culture (median fluorescence intensity = 41.3 at day 4). As expected (21), Fas expression was higher in T cells stimulated with anti-CD3 mAb (median fluorescence intensity = 85.1) compared with control cells at day 4. CD28 costimulation prevented the TCR-mediated up-regulation of Fas in $CD8^+$ cells (median fluorescence intensity = 39.02 at day 4). Importantly, Letal/CD3 costimulation resulted in marked reduction of Fas expression in 60% of cells (median fluorescence intensity = 14.9 at day 4) compared with control unstimulated $CD8^+$ lymphocytes or CD3/CD28 costimulated lymphocytes (Fig. 5*B*). To test whether Letal engagement protects $CD8^+$ lymphocytes from FasL-induced apoptosis, $CD8^+$ cells stimulated for 3 days with different ligands as above were exposed to anti-Fas/CD95 agonistic antibody EOS9.1, and (early) apoptosis was quantified by flow cytometry analysis of annexin-V staining. The percentage of annexin V-negative nonapoptotic cells was markedly higher among CD3/Letal-costimulated T cells compared with CD3/CD28-costimulated, CD3-stimulated, or control T cells (Fig. 5*C*). Therefore, Letal protects $CD8^+$ lymphocytes from Fas-mediated apoptosis and thus renders them resistant to suicidal, fratricidal, or tumor-induced killing (21).

DISCUSSION

Here, we show that human advanced ovarian carcinoma exhibiting improved outcome and stronger lymphocyte infiltration also show higher levels of the NKG2D ligand Letal. Cytotoxic lymphocytes sorted from these tumors were negative for CD28, but Letal exerted marked costimulatory properties on TCR-mediated proliferation of these cells. Moreover, Letal engagement protected $CD8^+$ T cells from apoptosis induced by TCR-dependent mitogenic signals, tumor death ligands, and genotoxic drugs. Because NKG2D is an important activating receptor for $CD8^+$ lymphocytes and NK cells in peripheral tissues, these results have marked implications for tumor immunosurveillance.

NK cells may be responsible for rejection of tumors at early stages of malignant transformation (10, 22). However, we found that $CD8^+$ cells are markedly more frequent than NK cells in advanced ovarian carcinoma. This finding suggests that immune surveillance against advanced ovarian carcinoma is mainly accomplished through expansion of tumor-specific CTLs. The presence of tumor-infiltrating T cells correlates with MHC class-I expression of tumor cells in ovarian cancer (17). We found significantly higher expression of Letal in

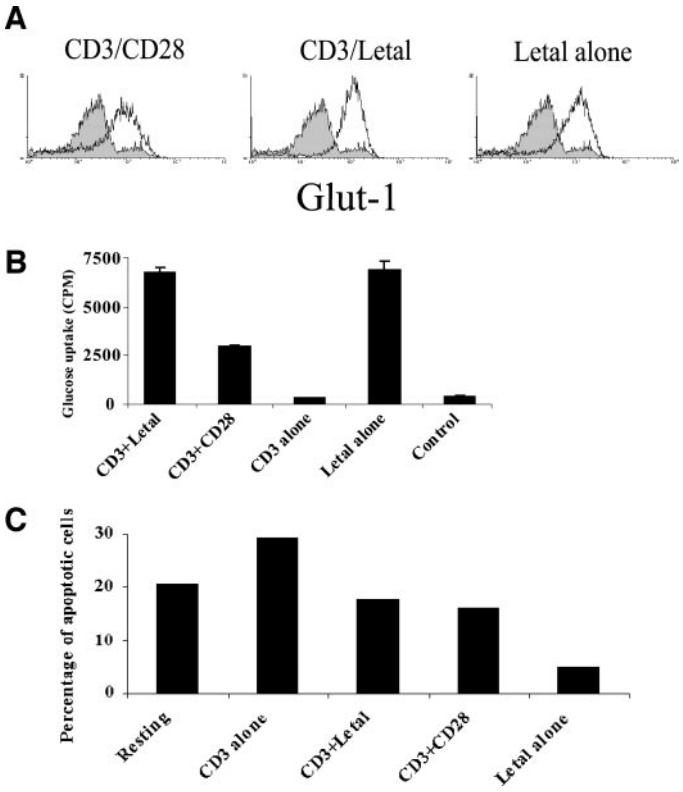

Fig. 4. Letal engagement increases the glycolytic flux and protects lymphocytes from genotoxic death. *A,* Letal alone or in combination with CD3 stimulation up-regulates Glut-1 as efficiently as CD3/CD28 costimulation in $CD8^+$ lymphocytes. *Shaded:* lymphocytes stimulated with CD3 alone. *B,* Letal alone or in combination with CD3 stimulation up-regulates glucose uptake by $CD8^+$ lymphocytes more efficiently than CD3/CD28 costimulation. *C,* Letal engagement protects $CD8^+$ lymphocytes from genotoxic drugs. Peripheral $CD8^+$ T cells were stimulated for 3 days with the indicated factors and then incubated with cisplatin. Note that results are expressed as percentage of apoptotic cells. All results are representative of at least three experiments.

tumor exhibiting accumulation of T cells, and we demonstrated a significant association between Letal expression and the presence of intratumoral T cells. Given the previously described role of Letal in enhancing the proliferation and function of TCR-activated $CD8^+$ cells, tumor-associated Letal emerges as an important costimulatory ligand promoting the expansion of tumor-specific CTLs in the context of MHC-I expression.

It is known that upon repeated stimulation with antigen, particularly in the presence of interleukin-2, T cells become susceptible to the induction of Fas-mediated apoptosis (20). Increased resistance of T cells to apoptosis is a necessary condition for the establishment of chronic inflammatory diseases and is required for the orchestration and endurance of sustained immune responses (23, 24). In lymph nodes, activation-induced T-cell apoptosis is inhibited by costimulatory signals provided by professional antigen-presenting cells through CD28, CD7, or some members of the tumor necrosis factor receptor family. Engagement of CD28 involves activation of mitogen-activated protein kinases extracellular signal-regulated kinase, p38, and c-Jun NH_2-terminal kinase, activation of nuclear factor-κB, up-regulation of Bcl-2, Bcl-x_L, and c-FLIP, and down-regulation of Fas (25–27). In the periphery, NKG2D serves as one of the most potent costimulatory receptors for $CD8^+$ effector lymphocytes. Engagement of NKG2D by Letal was found to markedly decrease expression of Fas and significantly reduced TCR activation-induced apoptosis. The survival-promoting effect of Letal was not restricted to the Fas pathway however, as Letal engagement protected lymphocytes also from genotoxic death such as induced by cisplatin. This finding has important implications for the effects of chemotherapy on antitumor immune response because activated lymphocytes become susceptible to toxic metabolites and cytotoxic chemotherapy has been shown to deplete tumor-reactive T cells (21). Collectively, Letal appears to play an important role in promoting the accumulation of intratumoral T cells by enhancing both their proliferation and their survival in the tumor microenvironment.

In lymphocytes, glycolytic metabolism may play a critical role in controlling cell survival. Withdrawal of exogenous survival factors results in a decline in cellular ATP, which is due, in part, to decreased expression of Glut-1, the major glucose transporter in lymphocytes (19). Major cell survival pathways have been reported to alter the metabolic response of lymphocytes to withdrawal of survival factors, sustain ATP production in mitochondria, increase glucose uptake, and/or enhance glycolysis in the absence of extracellular signals (19, 28). $CD8^+$ T-cell activation is accompanied by a dramatic increase in glucose uptake through up-regulation of Glut-1. It has been recently reported that CD3/CD28 T-cell costimulation increases glycolytic flux in a manner similar to that of the insulin receptor (16). Letal also induced a dramatic increase in glucose uptake and up-regulation of Glut-1. Thus, NKG2D engagement, similarly to CD3/CD28 costimulation, allows T cells to anticipate the energetic needs of a sustained immune response and appears to afford prosurvival signals through regulation of the glycolytic pathway. Interestingly, Letal signaling

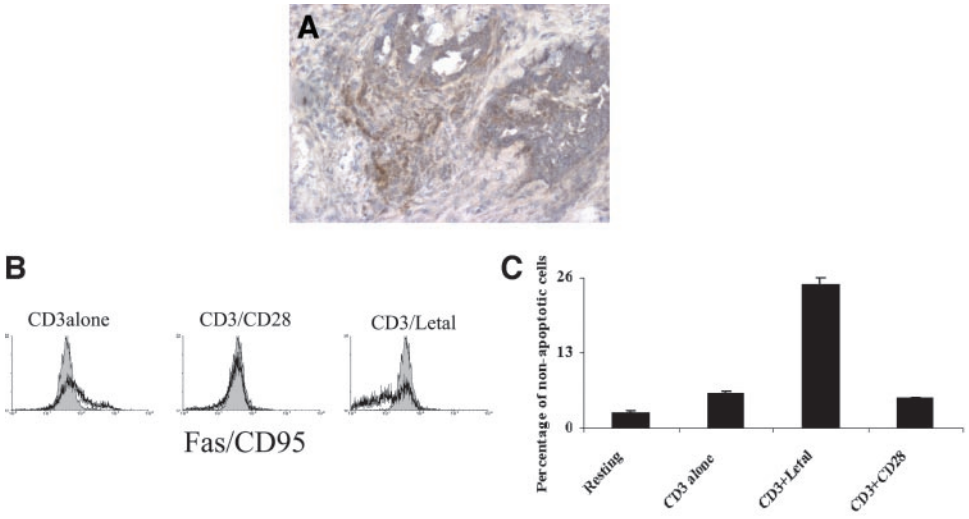

Fig. 5. Letal engagement protects lymphocytes from Fas-dependent apoptosis. *A,* select ovarian carcinoma specimens exhibit intense Fas ligand staining in tumor cells by immunohistochemistry. *B,* down-regulation of Fas/CD95 by peripheral blood lymphocytes upon CD3/Letal engagement. Lymphocytes were stimulated for 4 days with the indicated conditions, and Fas expression was analyzed by flow cytometry. *Shaded:* unstimulated $CD8^+$ cells at day 4. *C,* Letal stimulation induces resistance to Fas ligand-dependent apoptotic death. $CD8^+$ lymphocytes treated with the indicated factors for 3 days were exposed to agonistic anti-Fas antibody EOS9.1 that delivers an apoptotic signal to Fas-expressing cells. More than 25% of Letal-stimulated lymphocytes resist apoptosis after 18 h. A representative analysis of three experiments is shown. Note that results are expressed as percentage of nonapoptotic cells.

alone could trigger glucose up-take, thus the parallels and differences with the CD28 pathway remain to be established.

We have recently demonstrated that the presence of T cells infiltrating tumor islets is associated with dramatically longer survival and that optimal surgical debulking and intratumoral T cells are two robust and independent prognosticators in ovarian carcinoma (3). Here, we show that Letal emerges as an additional independent prognosticator of outcome in ovarian cancer. Together with tumor debulking and intratumoral T cells, expression of the NKG2D ligand defined a subset of patients with advanced ovarian cancer who experienced dramatically improved survival. Furthermore, in the intermediate prognosis group, in the presence of intratumoral T cells, expression of Letal by the tumor had the same impact on survival as optimal debulking. These data underscore the impact of immunosurveillance mechanisms on the natural course of ovarian cancer. Additional NKG2D ligands have been described, including MICA and MICB, as well as UL16-binding protein-1, UL16-binding protein-2, and UL16-binding protein-3, with relatively restricted expression in tissues. Given that antitumor response may vary depending on the level of NKG2D ligands available (10), investigation of other NKG2D ligands is warranted in ovarian cancer. These mechanisms may be relevant in other malignancies as well. In fact, we have previously reported a highly prevalent expression of Letal in colon cancer cell lines (13).

Important questions follow on the mechanisms accounting for failure of immune surveillance. It has been reported that engagement of soluble forms of NKG2D ligands causes down-regulation of NKG2D and, in turn, impairment of T-cell activation (29). We have found that glycosylphosphatidylinositol-specific phospholipase-D (GLPD1) mRNA levels were significantly higher in stage I or III than in benign or low malignant potential tumors (our unpublished observations), suggesting that different glycosylphosphatidylinositol-anchored NKG2D ligands (30) may be secreted by enzymatic cleavage *in vivo* and impair the expression of NKG2D in select tumors. Alternatively, the imunosurveillance pressure may eventually select out immunoresistant tumor variants that can escape CTL-mediated killing, induce T-cell apoptosis or unresponsiveness (anergy; Ref. 31) or simply divide faster than CTL can kill.

Recently, the efficacy of adoptive transfer of *ex vivo* expanded TILs has been documented in chemotherapy and immunotherapy-resistant metastatic melanoma (6), renewing interest in adoptive immunotherapy. The present findings show that Letal could be used as a costimulatory ligand to aid in the *ex vivo* preparation of tumor-reactive adoptive T cells that are apoptosis resistant. Such T cells may be derived directly from tumors and may be expanded using artificial APCs armed with Letal, as we showed above. Given that peripheral effector $CD8^+$ cells are mainly $CD28^{low/neg}$, such approach might offer significant advantage over CD28-based costimulation. Furthermore, Letal overexpression at tumor sites could promote the expansion of tumor-specific T cells and their protection from apoptotic signals, including chemotherapy.

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