ICAM-1 Has a Critical Role in the Regulation of Metastatic Melanoma Tumor Susceptibility to CTL Lysis by Interfering with PI3K/AKT Pathway

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Abstract

Human primary melanoma cells (T1) were found to be more susceptible to lysis by a Melan-A/MART-1–specific CTL clone (LT12) than their metastatic derivative (G1). We show that this differential susceptibility does not involve antigen presentation by target cells, synapse formation between the metastatic target and CTL clone, or subsequent granzyme B (GrB) polarization. Although PI-9, an inhibitor of GrB, was found to be overexpressed in metastatic G1 cells, knockdown of the *PI-9* gene did not result in the attenuation of G1 resistance to CTL-induced killing. Interestingly, we show that whereas T1 cells express high levels of intercellular adhesion molecule-1 (ICAM-1), a dramatically reduced expression was noted on G1 cells. We also showed that sorted ICAM-1$^+$ G1 cells were highly sensitive to CTL-induced lysis compared with ICAM-1$^-$ G1 cells. Furthermore, incubation of metastatic G1 cells with IFN-γ resulted in the induction of ICAM-1 and the potentiation of their susceptibility to lysis by LT12. More importantly, we found that the level of ICAM-1 expression by melanoma cells correlated with decreased PTEN activity. ICAM-1 knockdown in T1 cells resulted in increased phosphorylation of PTEN and the subsequent activation of AKT. We have additionally shown that inhibition of the phosphatidylinositol (3,4,5)-triphosphate kinase (PI3K)/AKT pathway by the specific inhibitor wortmannin induced a significant potentiation of susceptibility of G1 and ICAM-1 small interfering RNA–treated T1 cells to CTL-induced lysis. The present study shows that a shift in ICAM-1 expression, which was associated with an activation of the PI3K/AKT pathway, can be used by metastatic melanoma cells to escape CTL-mediated killing. [Cancer Res 2008;68(23):9854–64]

Introduction

Melanoma is an aggressive malignancy with poor prognosis owing to its resistance to chemotherapy. The identification of tumor-associated antigens (TAA) recognized primarily by CD8$^+$ T lymphocytes has led to vaccination strategies that induce or potentiate specific immune responses. However, cancer vaccine immunotherapy is not always followed by a successful clinical response (1). Metastatic tumors use various immune escape mechanisms, including loss or down-regulation of HLA class I molecules and antigen expression (2). Despite the expression of TAA, tumor eradication by the immune system is often inefficient (3). In this regard, it has been reported that metastatic cancer cells are more resistant to apoptosis than their poorly metastatic counterparts, which indicates that apoptotic pathways in metastatic cancer cells may be more highly dysregulated (4, 5).

The potential involvement of intercellular adhesion molecule-1 (ICAM-1 or CD54), a ligand of lymphocyte function–associated antigen-1 (LFA-1), in the interaction between CTL and target cells and thus in the regulation of CTL-mediated cytotoxicity has been well documented in a variety of studies (6–8). There is strong evidence that the engagement of LFA-1 on CTL by ICAM-1 on target cells is essential for T-cell activation and for directing the released of cytolytic granules into the tumor cells (9–11). In addition, because ICAM-1 is associated with other cell molecules, including MHC class I proteins, its engagement on target cells may lead to recruitment of MHC-I proteins to the contact area and enhanced presentation of cognate peptide MHC-I complexes to cytotoxic T cells (12). *In vivo* experiments indicate that ICAM-1 overexpression on tumor cells resulted in a reduced tumor growth rate, correlating with increased lysis by tumor-infiltrating lymphocytes (TIL; refs. 13–15). Studies using LFA-1–deficient mice underlined the pivotal role for the LFA-1/ICAM-1 interaction in the rejection of immunogenic tumors but not for clearance of systemic infections (16), suggesting their particular role in antitumor immunity. In addition, several studies emphasized the crucial role of ICAM-1 in the activation of CD8$^+$ T cells in the absence of costimulation provided by CD80 and CD86 molecules on tumor cells (6). It should be noted that ICAM-1 may function as a cell surface receptor capable of initiating intracellular signaling. In this regard, several studies on human endothelial cells showed that although the cytoplasmic tail of ICAM-1 lacks intrinsic enzymatic activity, engagement of ICAM-1 triggers several signaling pathways, including activation of Src family kinase leading to

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phosphorylation of actin-associated proteins (17), including p44/p42 mitogen-activated protein kinase (MAPK; ref. 18), and p38 MAPK (19). Several studies showed that ICAM-1 expression correlated with melanoma thickness and suggested that it may contribute to the metastatic potential of melanoma cells (20, 21). However, the contribution of this cell adhesion molecule in the regulation of CTL-induced cell death remains unresolved.

A recent study has revealed that, in a redox-dependent manner, ICAM-1 regulated the expression of PTEN (phosphatase and tensin homologue deleted from chromosome 10), a tumor suppressor and negative regulator of the phosphatidylinositol (3,4,5)-triphosphate kinase (PI3K)/protein kinase B (AKT) pathway that plays an important role in tumorigenesis (22, 23). Obviously, the PTEN/PI3K/AKT pathway is one of the important survival factors that contribute to resistance to apoptosis signals (24, 25). Indeed, this pathway has been implicated in the chemoresistance observed in a wide range of human cancers, including melanoma, breast, lung, prostate, and ovarian tumors (26, 27). In this regard, cell lines with defective PTEN have alterations in cell cycle regulation and a defective apoptotic response (28). AKT has been shown to interfere with apoptotic signaling through various mechanisms (24, 29, 30). Although the PTEN/PI3K/AKT pathway has been reported to be involved in the resistance of human cancer to chemotherapy and radiation therapy, little is known about its involvement in the regulation of tumor cell susceptibility to CTL-mediated lysis.

The present study examines the molecular basis of the differential susceptibility of primary and metastatic melanoma cells to specific lysis by autologous CTL clones. Our results show that down-regulation of ICAM-1 expression by metastatic melanoma cells correlated with increased PTEN activity, activation of the PI3K/AKT pathway, and the subsequent development of metastatic melanoma resistance to CTL. The current study emphasizes that in addition to its potential role in CTL activation, ICAM-1 on target cells may sensitize those cells to CTL-mediated killing by interfering with activation of the PTEN/PI3K/AKT pathway through PTEN dephosphorylation.

Materials and Methods

Antibodies and reagents. Antibodies directed against caspase-3 [8G10, rabbit monoclonal antibody (mAb)], phospho-PTEN$^{(Ser380/Thr382/Thr383)}$ (rabbit polyclonal antibodies), PTEN (138G6, rabbit mAb), phospho-AKT$^{(Ser473)}$ (mouse mAb), and AKT (rabbit polyclonal antibodies) were purchased from Cell Signaling Technology. Bid (FL195, rabbit IgG) and actin (C11, goat IgG) were purchased from Santa Cruz Biotechnology. Anti-serpin PI-9 (7D8, mouse mAb) was from Cayman Chemical. Recombinant human granzyme B (GrB) was purchased from Alexis Biochemicals. Anti-LFA-1, anti-CD2, anti-LFA-3, anti-ICAM-1, and anti-HLA-A2 (MA2.1) mAbs were reported previously (31). Mouse mAb directed against Melan-A (A103, IgG1) was purchased from Dako and anti-GrB mAb was from Caltag Laboratories (Invitrogen).

Synthetic peptides. The Melan-A peptides 26-EAAGIGILTV-35 and the decamer analogue ELAGIGILTV were purchased from Genepep S.A. Purity (>70%) was controlled by reversed-phase high-performance liquid chromatography. Lyophilized peptides were dissolved in DMSO at 10 mg/mL and stored at −80°C.

Tumor cell lines and CTL clones. T1 and G1 melanoma cell lines were derived, respectively, from the primary lesion and from the metastatic lymph node of the same patient (FON) as previously described (32). The melanoma cells were cultured in RPMI 1640 supplemented with 5% FCS, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate at 37°C in a humidified atmosphere containing 5% CO_2.

The LT12 CTL clone, specific for Melan-A_{25-36} peptide, was isolated from autologous TILs as described previously (32) and cultured at 37°C (5% CO_2) in complete medium (RPMI 1640 with GlutaMAX; Invitrogen) supplemented with 1% sodium pyruvate (Invitrogen), 8% human serum (Institut Jacques Boy), and recombinant interleukin-2 (150 units/mL) in the presence of the T1 autologous tumor cell line and irradiated LAZ and allogeneic peripheral blood mononuclear cells. PHA was added (1 μg/mL).

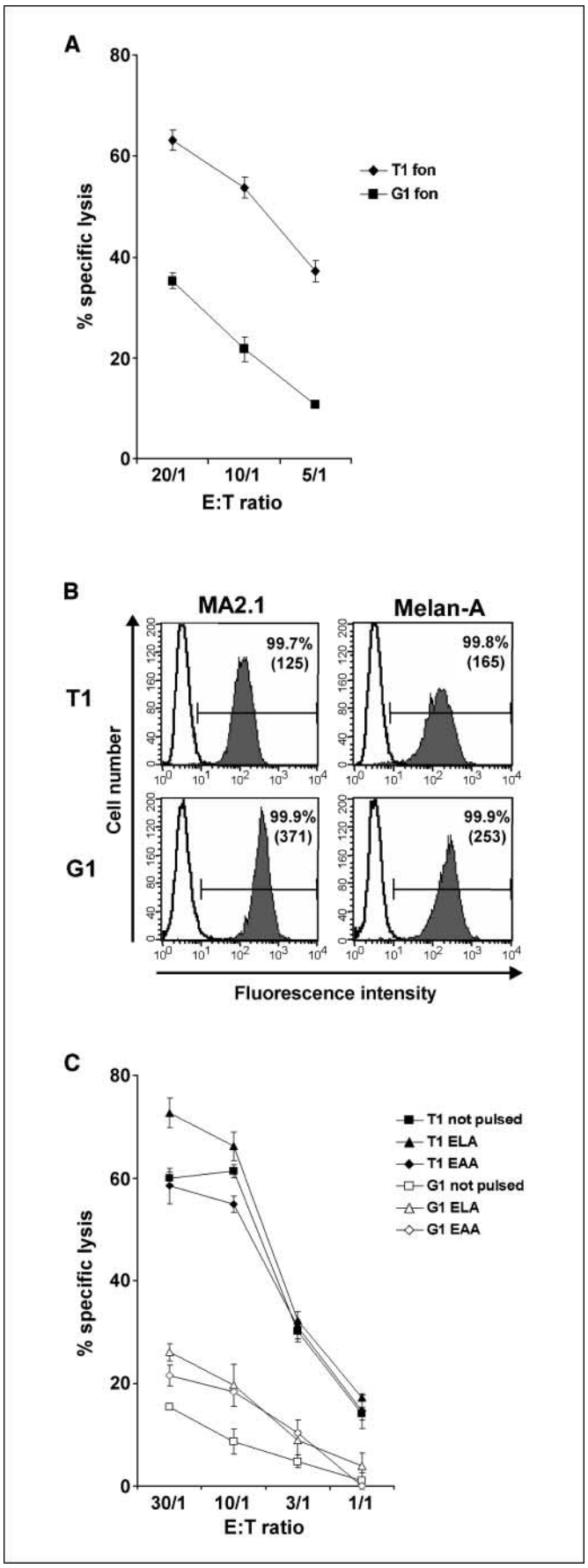
Cytotoxic activity assay. The cytotoxic activity of the CTL clone was measured by a conventional 4-h ^{51}Cr release assay by using triplicate cultures in round-bottomed 96-well plates. Several E:T ratios were used on 1,000 target cells per well. Supernatants were then transferred to LumaPlate-96 wells (Perkin-Elmer), dried down, and counted on a Packard TopCount NXT. Percent specific cytotoxicity was calculated conventionally. In peptide pulsing experiments, target cells were incubated for 1 h with synthetic peptide (10 μmol/L) at 37°C before adding effector cells. In blocking experiments, effector cells were incubated for 1 h with anti-LFA-1 mAb at room temperature before adding target cells.

Flow cytometry analysis. For cell surface staining, cells (3×10^5) were incubated with anti-ICAM-1, anti-HLA-A2 (MA2.1), anti-LFA-1, anti-CD2, and anti-LFA-3 mAbs or isotypic control antibodies for 30 min at 4°C followed by FITC-conjugated goat anti-mouse antibody. For intracellular staining, cells were fixed with 4% (w/v) paraformaldehyde solution (Sigma) in 1× PBS for 10 min at room temperature, washed, and then permeabilized by 0.1% (w/v) saponin (Sigma)/0.1% (w/v) BSA solution in 1× PBS for 20 min and washed in 1× PBS. The indirect immunofluorescence procedure was performed as previously described (33). Primary mouse anti-Melan-A antibody was applied (1:40 dilution in 1× PBS, 0.1% BSA, 0.1% saponin) at room temperature followed by three washes in 1× PBS and incubation with FITC-conjugated goat anti-mouse antibody for 30 min. After staining, cells were resuspended in 1× PBS and analyzed on a FACSCalibur flow cytometer, and data were processed using CellQuest software (Becton Dickinson).

Confocal scanning immunofluorescence microscopy. T1/G1 cells cultured on poly-L-lysine coverslips (MatTek Corp.) were coincubated with LT12 CTL clone at 2:1 E:T ratio for 45 min. For analysis of raft distribution, LT12 clones were incubated with 8 μg/mL of cholera toxin–Alexa Fluor conjugate (Molecular Probes, Invitrogen) in 1× PBS for 35 min at 4°C. After washing, CTB-loaded LT12 clones were dropped onto target cells previously cultured on poly-L-lysine–coated coverslips. Cells were washed with 1× PBS, fixed with 4% (w/v) paraformaldehyde solution in 1× PBS for 1 h, and then permeabilized with SDS (0.1% w/v in 1× PBS) for 10 min followed by blocking with 10% fetal bovine serum (FBS) in 1× PBS for 20 min. The fixed cells were stained with anti-ICAM-1 (CD54) mAb or anti-GrB mAb and then with a goat anti-mouse secondary mAb coupled to Alexa Fluor 546 (red) or Alexa Fluor 488 (green) secondary antibody (Molecular Probes, Invitrogen). Nuclear staining was performed with TO-PRO-3 (far red; Molecular Probes, Invitrogen). Coverslips were mounted on glass slides using a drop of Vectashield hard set (Vector Laboratories, Inc.). The fluorescence was examined under an LSM 510 confocal microscope (Zeiss) as previously described (34). Polarization of cytotoxic granules was defined by accumulation of GrB in the contact area between CTLs and tumor cells.

Cell sorting for ICAM-1^{neg}, ICAM-1^{bright}, and ICAM-1^{high} G1 tumor cells. G1 cells were collected, stained with anti-mouse ICAM-1 mAb and then with a goat anti-mouse secondary antibody conjugated to FITC, and sorted by a FACSVantage SE cell sorter based on ICAM-1 intensity.

Western blot analysis. Total cellular extracts were prepared by lysing cells in ice-cold buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% CHAPS, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin]. Equivalent protein extracts (30–50 μg) were denatured by boiling in SDS and β-mercaptoethanol, separated by SDS-PAGE, and transferred onto nitrocellulose membranes (Pierce/Perbio). The efficiency of the electrotransfer was assessed by Ponceau Red staining. Blots were blocked for 1 h with TBS containing 5% nonfat dry milk and probed with appropriate antibody for 3 h [anti-phospho-AKT (mouse mAb), anti-AKT (rabbit polyclonal antibody), anti-phospho-PTEN (rabbit polyclonal antibody), anti-PTEN (rabbit mAb), and anti-actin (goat polyclonal antibody)] or overnight [anti-caspase-3 (rabbit mAb)]. After washing, blots



were incubated with appropriate horseradish peroxidase–conjugated secondary antibody. The complexes were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce/Perbio).

Small interfering RNA transfection. The small interfering RNAs (siRNA) used were designed with the Qiagen RNA interference designer tool for specific down-regulation of serpin PI-9 and ICAM-1. The two sequences used were serpin PI-9 [CGCUUAUGCUACUUAAUCA dTdT (S1) and CCGUUUGACGAAACAUACA dTdT (S2)] and ICAM-1 [GGAGCUUC-GUGUCCUGUAU dTdT (S1) and GAUGGGCAGUCAACAGCUA dTdT (S2)] together with a negative control siRNA targeting luciferase purchased from Sigma-Proligo. Subconfluent cells were transfected with siRNA in Opti-MEM using the jetSI-ENDO reagent (from Polyplus-transfection Technologies) according to the manufacturer's instructions.

Loading of GrB. Analysis of tumor cell sensitivity to GrB was performed as previously described (35). Briefly, cells were plated on coverslips at a density of 1.5×10^5 per well in six-well plates. Forty-eight hours later, cells were loaded with recombinant GrB using sublytic doses of the pore-forming protein streptolysin O (SLO; Sigma). Briefly, SLO (2 μg/mL) was preactivated by incubating for 30 min at room temperature in 1× PBS containing 1 mmol/L DTT. Cells were then washed in serum-free medium followed by dropwise addition of 150 μL of RPMI 1640 containing 100 nmol/L GrB to cell monolayers. Wells were flooded 15 min later with 1.5 mL of RPMI 1640 with 5% FBS. Early apoptotic events were evaluated 3 h after loading GrB with $Dioc_6(3)$ and propidium iodide labeling and were analyzed on a FACSCalibur flow cytometer, and data were processed using CellQuest software.

Results

The differential susceptibility of primary and metastatic melanoma cells to CTL-induced lysis does not interfere with antigen presentation. Using the autologous CTL clone LT12, which is specific for Melan-A/MART-1 peptide (26-EAAGIGILTV-35, hereafter Melan-A) in the HLA-A2 context, we showed that metastatic G1 cells were less sensitive to CTL-mediated cell death even at a high E:T ratio than T1 primary melanoma cells, which displayed a strong sensitivity (Fig. 1*A*). We next asked whether the G1 cell resistance to killing by CTL interferes with impaired expression of Melan-A protein coding for antigen or with the loss of HLA-A2 molecules by these cells. Specific staining of HLA-A2 extracellular molecules and Melan-A intracellular protein showed that T1 and G1 cells expressed both of these proteins, ruling out their involvement in the resistance of G1 metastatic cells to CTL-induced killing (Fig. 1*B*). To examine if the decreased susceptibility to CTL-mediated killing depended on the amount of antigen at the cell surface, we investigated the lytic potential of the LT12 clone in response to T1 and G1 cells pulsed either with natural or modified (ELAGIGILTV) Melan-A peptide. A differential susceptibility to CTL killing between G1 and T1 cells, pulsed or not pulsed with peptides,

Figure 1. The differential susceptibility to CTL-induced lysis does not involve Melan-A antigen expression. *A,* the lytic activity of the LT12 CTL clone toward T1 and G1 tumor cells was assessed in a standard 4-h chromium release assay at various E:T ratios as indicated. Percent specific lysis was calculated conventionally. *B,* cell surface expression of HLA class I molecules and intracellular expression of Melan-A protein by T1 and G1 cells were determined by immunofluorescence analysis by fluorescence-activated cell sorting (FACS) using anti-HLA-A2 (MA2.1) and anti-Melan-A (*filled*) mAbs. Isotypic IgG1 (negative control) were included (*open*). *Numbers,* percentages of positive cells; *numbers in brackets,* mean fluorescence intensity of specific staining. *C,* the lytic activity of the LT12 CTL clone toward T1 and G1 tumor cells, pulsed or not pulsed with the natural peptide (*EAAGIGILTV*) or with the modified peptide (*ELAGIGILTV*), was assessed in a standard 4-h chromium release assay at various E:T ratios as indicated. Percent specific lysis was calculated conventionally. Representative data of more than three independent experiments are shown.

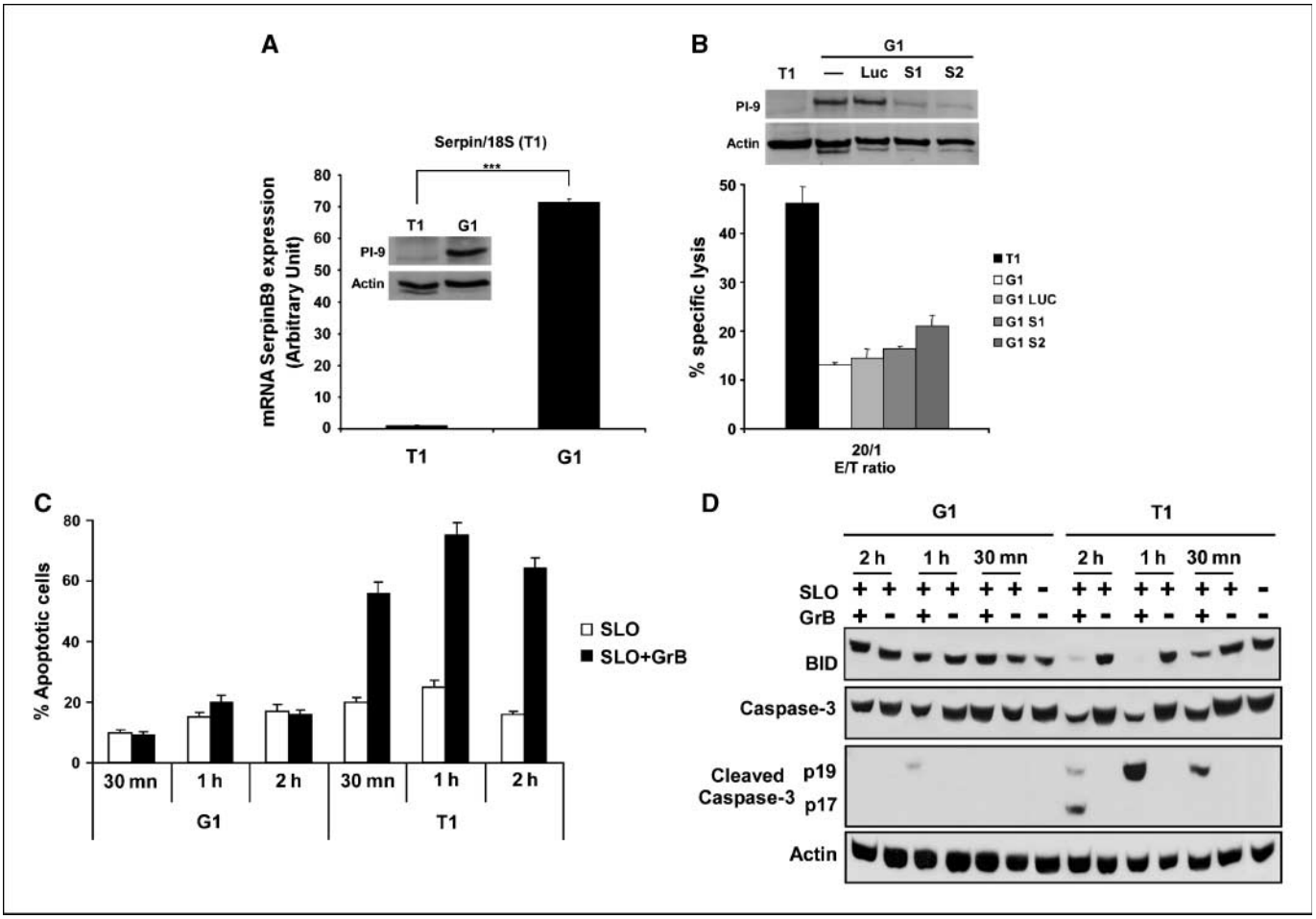


Figure 2. Effect of modulation of serpin PI-9 on CTL-induced killing of T1 and G1 cells. *A,* the expression of *serpin PI-9* gene in metastatic resistant G1 and primary sensitive T1 cell lines was assessed by quantitative RT-PCR. *Columns,* mean of three independent determinations; *bars,* SD. ***, $P < 0.001$. Experimental values were normalized to those for the *18S* gene. *Top,* PI-9 expression in T1 and G1 cells was determined by Western blot analysis using anti-serpin PI-9 mAb (clone 7D8). Actin was used as the protein level control. *B,* knockdown of serpin PI-9 in G1 cells was performed with two siRNAs (S1 or S2) for 72 h. A negative control siRNA (*Luc*) targeting luciferase was included. The lytic activity of the LT12 CTL clone toward these target cells was assessed in a standard 4-h chromium release assay at 20:1 E:T ratio. *Top,* serpin PI-9 expression was determined by Western blot analysis using anti-serpin PI-9 mAb (clone 7D8). Actin was used as the protein level control. *C* and *D,* T1 and G1 tumor cells were incubated with SLO (2 μg/mL) alone or in combination with recombinant human GrB (100 nmol/L) for the indicated times. *C,* apoptosis was assessed by flow cytometry using $Dioc_6(3)$ and propidium iodide staining. *D,* whole-cell lysates (30 μg) were subjected to SDS-PAGE, blotted, and probed with antibodies as indicated. The decrease in Bid expression indicates cleavage and activation of the protein, and the increase in p19 and p17 expression indicates cleavage of the procaspase-3 (p37). Actin was used as the protein level control. Representative data of several independent experiments are shown.

was always observed even if a weak increase in lysis with the analogous peptide was observed in both cells (Fig. 1*C*). This indicates that the resistance of G1 metastatic cells to CTL lysis is not associated with an alteration of Melan-A antigen presentation at cell surface.

Overexpression of serpin PI-9 by metastatic melanoma cells. Given that LT12-induced cell lysis is mediated by perforin/GrB and in light of the key role of PI-9 in the inhibition of this lytic pathway, we investigated PI-9 expression in T1 primary and G1 metastatic melanoma cells. For this purpose, we carried out real-time quantitative reverse transcription-PCR (RT-PCR) analysis. As shown in Fig. 2*A*, although *PI-9* gene transcription was weakly detectable in T1 cells, it was up-regulated (70 times higher) in G1 cells. These observations were confirmed by Western blot analysis using an anti-human PI-9 mAb (Fig. 2*A, top*), indicating a high expression of PI-9 protein in G1 cells. Unexpectedly, the inhibition of PI-9 expression in G1 cells using specific siRNAs (S1 and S2) was only accompanied by a marginal increase in the susceptibility of G1 cells to CTL killing compared with G1 cells transfected with the siRNA Luc control (Fig. 2*B*). Using recombinant GrB, we also showed that T1 and G1 cells were differentially killed (75 ± 4% for T1 versus 20 ± 2% for G1; Fig. 2*C*). Such differential susceptibility to GrB-induced apoptosis correlated with Bid and caspase-3 cleavage in these cells (Fig. 2*D*). These results suggest that the resistance of metastatic G1 cells to the LT12 clone was not associated with PI-9 overexpression but presumably with some events downstream of GrB that protected these cells from destruction by the CTL clone.

Normal synapse formation despite low ICAM-1 expression in metastatic melanoma cells. The loss of adhesion molecules by metastatic cells has been well documented. Therefore, we investigated the expression of ICAM-1, ICAM-2, and LFA-3 on T1 and G1 cells using immunofluorescence staining. Results depicted in Fig. 3*A* indicated that whereas ICAM-1 was expressed at high levels on T1 cells compared with G1 cells, ICAM-2 and LFA-3 were expressed at similar levels by both cells. In contrast, weak expression of ICAM-1 was observed on G1 cells (22%). It is worth noting that LFA-1 and CD2 were expressed on the LT12 clone.

It was reported that ICAM-1/LFA-1 interaction is an essential requirement for triggering CTL adhesion and for directing cytolytic granule release to the surface of target cells. Confocal microscopy analysis indicates that ICAM-1 accumulates in the contact area between LT12 clone and T1 cells but not G1 cells, which display a low level of ICAM-1 (Fig. 3*B*). However, analysis of synapse formation using cholera toxin B–labeled lipid rafts, which accumulated in the contact area between effector and target cells (Fig. 3*B*), indicated that the LT12 clone formed equivalent stable conjugates with G1 and T1 cells with similar lipid raft coalescence (for $n = 100$ analyzed conjugates, 90% exhibited raft relocalization). Next, we examined the polarization of cytotoxic granules in the CTL clone LT12 cocultured with either T1 or G1 cells. GrB accumulation in the contact area between effector and tumor cells was analyzed by confocal microscopy. Data shown in Fig. 3*C* indicate that polarization of cytotoxic granules occurred in a similar manner in LT12 when cocultured either with T1 or G1 cells. For 100 analyzed LT12 cells, 86% and 85% of LT12 cells formed conjugates with T1 and G1 cells, and 77% and 71% of analyzed LT12 cells exhibited GrB relocalization with T1 and G1 cells, respectively. These observations suggest that the decreased susceptibility of metastatic cells to killing is not likely a consequence of a defect in synapse formation or an alteration of polarization of cytotoxic granules despite the significant down-regulation of ICAM-1 in G1 cells.

The modulation of ICAM-1 expression in metastatic G1 cells correlates with the modulation of their susceptibility to CTL- and GrB-induced lysis. To get more insight into the potential role of ICAM-1 in controlling target susceptibility to CTL lysis, G1 cells were first sorted and selected into three subpopulations according to the expression level of ICAM-1 using FACSVantage (Fig. 4*A, top*). Data depicted in Fig. 4*A* indicate that the lytic capacity of LT12 correlates with the level of ICAM-1 expressed by G1 cells. When LFA-1 was neutralized by a specific mAb, T1 and G1 cell lysis by LT12 was abrogated. Similar results were obtained using recombinant GrB. ICAM-1–positive G1 cells displayed a strong sensitivity to recombinant GrB treatment comparable with that of T1 cells (Fig. 4*A*). Interestingly, we showed that up-regulation of ICAM-1 expression by G1 cells following IFN-γ treatment (Fig. 4*B, top*) resulted in a high potentiation of G1 lysis by LT12 (Fig. 4*B*). Such lysis was inhibited by anti-LFA-1 mAb. In parallel, the incubation of G1 cells with IFN-γ was also accompanied by an increase in their susceptibility to GrB-induced apoptosis (Fig. 4*B*). These data further confirm that induction of ICAM-1 on resistant metastatic cells is sufficient to restore their lysis.

The level of ICAM-1 expression by melanoma cells regulates the activation of PI3K/AKT pathway. It has been recently reported that ICAM-1 plays a role in the regulation of PTEN expression (22). We asked, therefore, whether a link exists between ICAM-1 expression, PTEN and AKT activation, and target susceptibility to CTL-induced lysis. For this purpose, we assessed PTEN phosphorylation (at Ser^{380}, Thr^{382}, and Thr^{383}), which restricts PTEN activity. Figure 5*A* shows that G1 cells displayed a high expression and phosphorylation of PTEN and AKT compared with T1 cells. In G1 cells highly expressing ICAM-1, reduced phosphorylation and expression of PTEN and AKT were observed. This was confirmed by the ratios of phosphorylated to total PTEN and AKT between cell types. Inhibition of ICAM-1 expression in T1 cells using specific siRNAs (S1 and S2) was accompanied by an increase in phosphorylation of PTEN and AKT and resulted in a decrease of cell susceptibility to CTL-mediated killing compared with T1 cells transfected with the siRNA Luc control (Fig. 5*B*). These data suggest that loss of ICAM-1 expression correlates with increased PTEN phosphorylation, which inhibits its activity and the subsequent activation of PI3K/AKT pathway. More importantly, inhibition of the PI3K/AKT pathway in T1 and G1 cells using wortmannin, a PI3K-specific inhibitor, resulted in an inhibition of AKT phosphorylation in G1 cells and a significant increase in their susceptibility to CTL killing (Fig. 5*C*). When ICAM-1 siRNA-treated T1 cells were cultured in the presence of wortmannin, a restoration of their sensitivity to CTL-mediated killing was also observed (Fig. 5*D*). These data strongly suggest that the PI3K/AKT pathway plays a critical role in the control of metastatic target susceptibility to specific lysis.

ICAM-1 down-regulation in IFN-γ–treated G1 cells reverses the IFN-γ–induced potentiation of lysis. To further strengthen the correlation between ICAM-1 expression and lysis, and ICAM-1 expression and PTEN activity, we investigated whether the ICAM-1 down-regulation by siRNA in IFN-γ–treated G1 cells would result in reversing the IFN-γ–induced higher lysis and the PTEN/AKT status. The results shown in Fig. 6 indicate that ICAM-1 overexpression in IFN-γ–treated G1 cells is accompanied by a decrease in PTEN and AKT phosphorylation compared with untreated G1 cells (Fig. 6*A*). Furthermore, the down-regulation of ICAM-1 in IFN-γ–treated G1 cells restored the phosphorylation of PTEN and AKT to a level comparable with untreated G1 cells. Data depicted in Fig. 6*B* and *C*, respectively, show that ICAM-1 siRNA significantly reduced both GrB-induced lysis and CTL-induced lysis of IFN-γ–treated G1 cells. These data suggest that induced ICAM-1 on resistant metastatic G1 cells plays a role in the restoration of their susceptibility to CTL- and GrB-mediated lysis.

Discussion

It is well established that malignant transformation is associated with genetic alterations, providing tumor cells with different mechanisms that allow them to escape immune surveillance and, in particular, to counteract effector T-cell responses. However, little is known about the mechanisms involved in the acquisition of metastatic cell resistance to CTL-mediated lysis. Here, we provide evidence that the decreased susceptibility of metastatic melanoma to CTL-induced killing involves down-regulation of ICAM-1 expression by these cells. Similar results were obtained in additional primary and metastatic melanoma cells derived from a same patient (data not shown). Lee and colleagues (36) have reported that although both primary and metastatic cells induced antitumor CTLs in syngeneic hosts, the metastatic cells, unlike the primary cells, were resistant to CTL lysis in a mouse prostate cancer model. Loss of expression of HLA class I antigens has been shown in a wide variety of tumors and is considered to be one of the mechanisms whereby tumors escape T-cell surveillance (37, 38). However, Takenoyama and colleagues (39) have shown that although HLA class I molecules were maintained in primary and metastatic human lung carcinoma cells established from the same patient, only the primary cells were lysed by CTL clone. This agrees with our previous report indicating that HLA class I expression was maintained in G1 and T1 cells (40). Moreover, to elude immune recognition, tumor cells down-regulate or even lose TAA completely (41, 42). In this context, Kurnick and colleagues (43) described immunoselected melanoma cells that expressed low levels of the antigen Melan-A/MART-1 after coculture with CTL

due to the down-regulation of the antigen via the silencing of its promoter. The data presented here show that the resistance of G1 cells to CTL-mediated killing was independent of cellular antigen processing or presentation, indicating that the antigen recognized by CTL clone LT12 was maintained in the tumor cells throughout tumor progression. We also provided data suggesting that the peptide density was not associated with the differential susceptibility of primary and metastatic melanoma cells to CTL-mediated lysis.

Several reports emphasize that CTL recognize both primary and metastatic tumor cell lines but may use different mechanisms to lyse these targets. In this regard, Bergmann-Leitner and Abrams (44) reported that a mutated *ras*-specific CTL clone attacked the primary colon carcinoma cell line by both the perforin-mediated

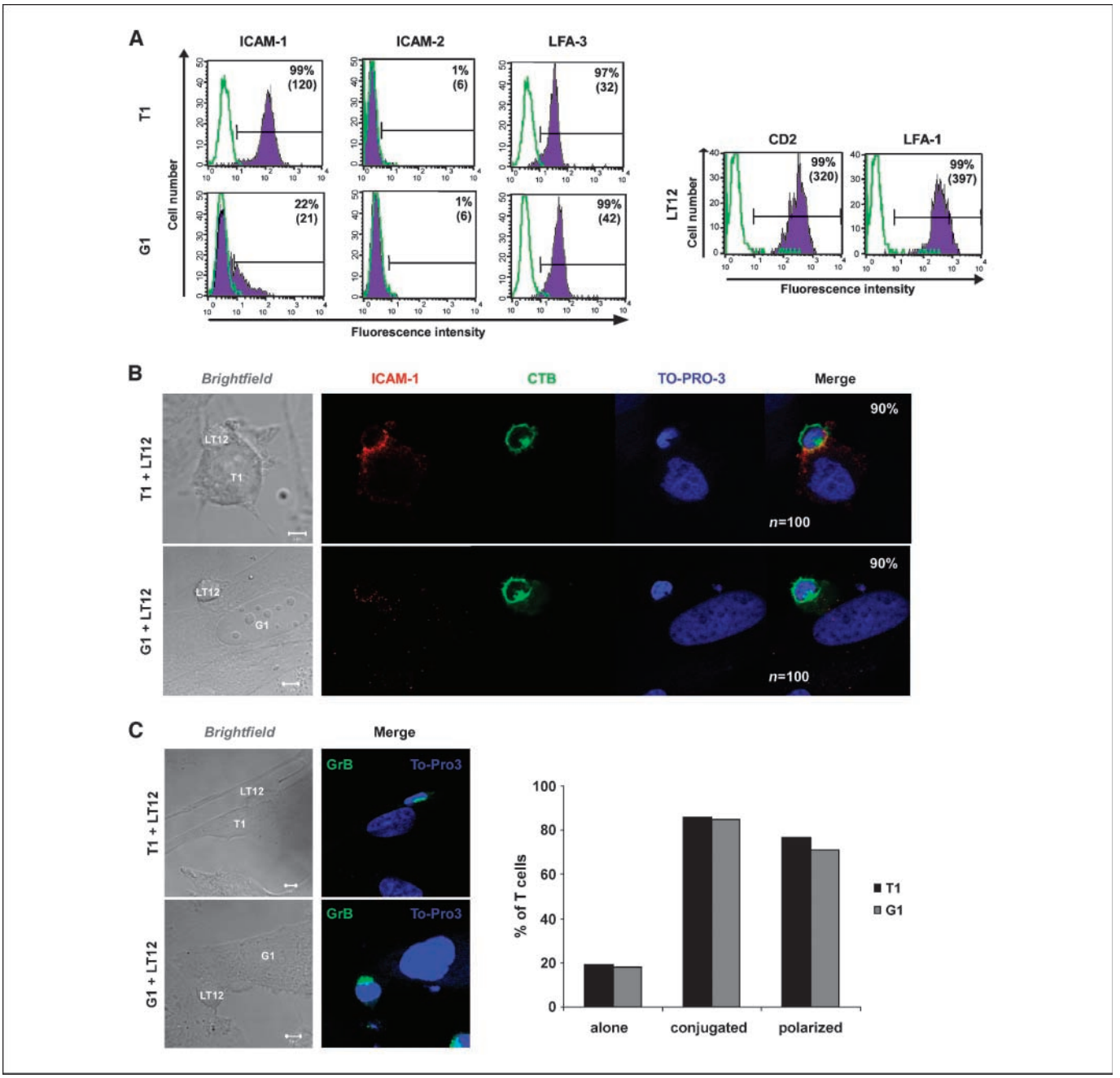


Figure 3. Immunologic synapse formation between LT12 clone and T1/G1 tumor cells. *A,* surface expression of adhesion molecules on T1/G1 target tumor cells and their respective ligands on LT12 CTL clone was determined by immunofluorescence analysis using anti-ICAM-1, anti-ICAM-2, anti-LFA-3, anti-LFA-1, and anti-CD2 (*filled*) mAbs. Isotypic IgG1 (negative control) was included (*open*). *Numbers,* percentages of positive cells; *numbers in brackets,* mean fluorescence intensity of specific staining. *B,* analysis of ICAM-1 recruitment (*red*) and aggregation of lipid rafts labeled by CTB (*green*) in the contact area between the LT12 clone and T1 or G1 cells after 45 min of coculture was assessed by confocal microscopy. Nuclei were stained with TO-PRO-3 (*blue*). *Bars,* 5 μm. Values indicate percentage of T cells with CTB aggregation in the contact area on $n = 100$ conjugates. *C, left,* granule polarization, as defined by the accumulation of GrB staining in the contact area between LT12 effector and T1 or G1 tumor cells after 45 min of coculture, was analyzed by confocal microscopy using a specific mAb (*green*). Nuclei were stained with TO-PRO-3 (*blue*). *Right,* percentages of CTL displaying GrB relocalization in the contact area of the conjugates. Experiments were repeated more than three times and representative data are shown.

and Fas/FasL-mediated pathways; however, the CTL clone attacked the metastatic colon carcinoma cell line that was derived from the same patient only by perforin-based pathway. In our experimental system, we have previously shown that neither T1 cells nor G1 cells expressed Fas or FasL at their surface. In addition, we showed that although tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) receptors were expressed by both T1 and G1 cells, G1 cells were resistant to TRAIL-mediated apoptosis (45). Because LT12 uses the GrB cytotoxic pathway to kill both T1 and G1 cells, the putative involvement of death receptors in the differential susceptibility of these cells is therefore ruled out. It is interesting to note that tumor cells resist CTL-induced killing through direct interference with the perforin/granzyme cytotoxic pathway. Therefore, we asked whether the serine inhibitor PI-9, which inhibits GrB, was involved in the acquisition of G1 metastatic cell resistance (46). Unexpectedly, we found that although PI-9 is up-regulated in resistant metastatic cells, silencing of PI-9 by siRNA did not attenuate this resistance to CTL-induced killing, suggesting that serpin PI-9 expression was not associated with the G1 resistance to specific lysis. Our data suggest that melanomas may

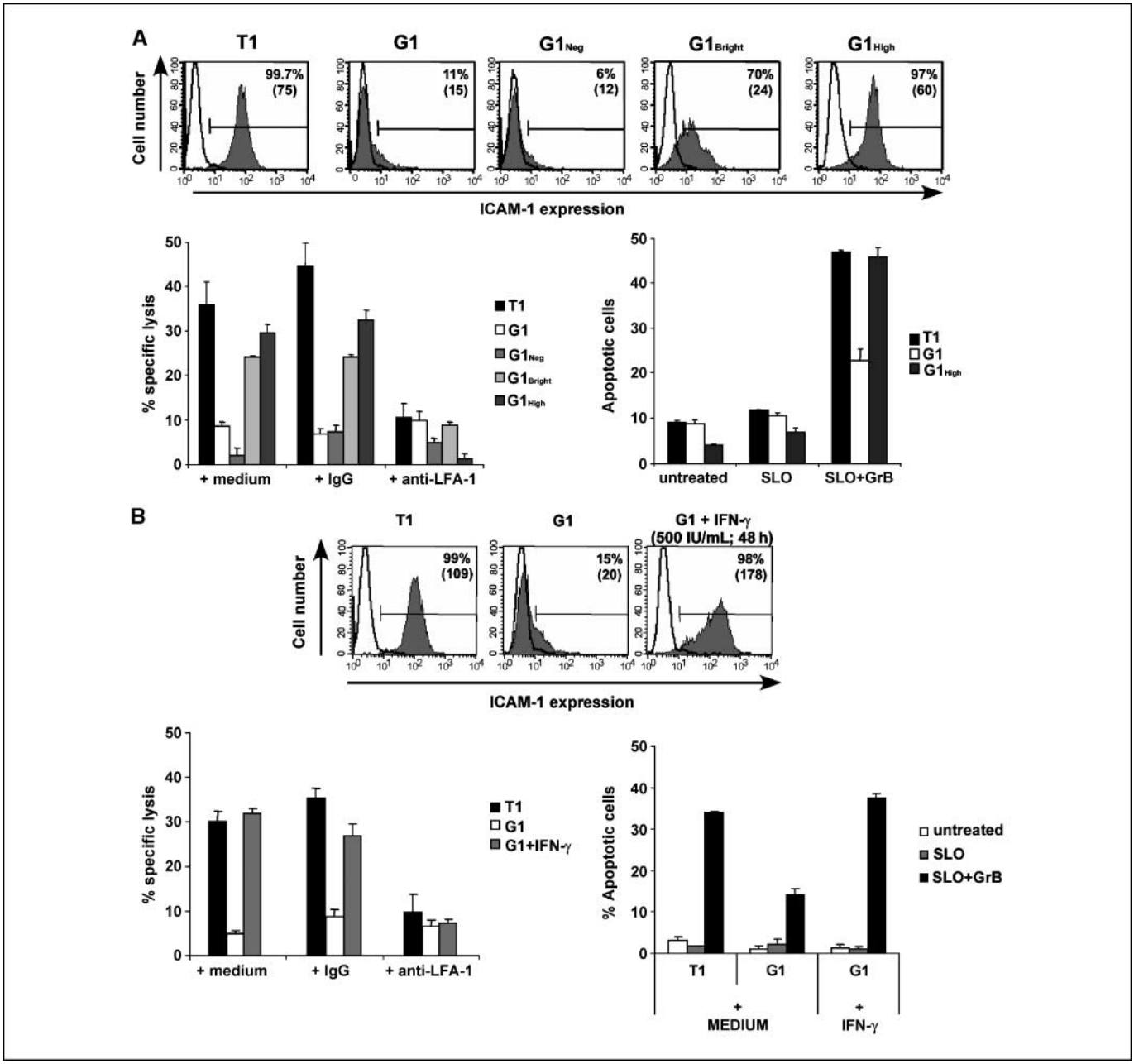


Figure 4. Consequences of ICAM-1 expression modulation on the lysis of T1 and G1 cells. *A* and *B, top,* surface expression of ICAM-1 on T1/G1 tumor cells, G1-sorted subpopulations, and IFN-γ–treated G1 cells was determined by FACS analysis using a specific mAb (*filled*) or isotypic control (*open*). *Numbers,* percentages of positive cells; *numbers in brackets,* mean fluorescence intensity of specific staining. *Bottom left,* the lytic activity of the LT12 CTL clone toward target tumor cells was assessed in a standard 4-h chromium release assay at 20:1 E:T ratio. The LT12 clone was preincubated or not for 1 h with saturating concentration of anti-LFA-1 mAb or an IgG1 control. *Bottom right,* tumor cell lines were incubated with SLO (2 μg/mL) alone or in combination with recombinant GrB complex (100 nmol/L) for 3 h. Apoptosis was assessed by $Dioc_6(3)$ and propidium iodide staining. Representative data of more than three independent experiments are shown.

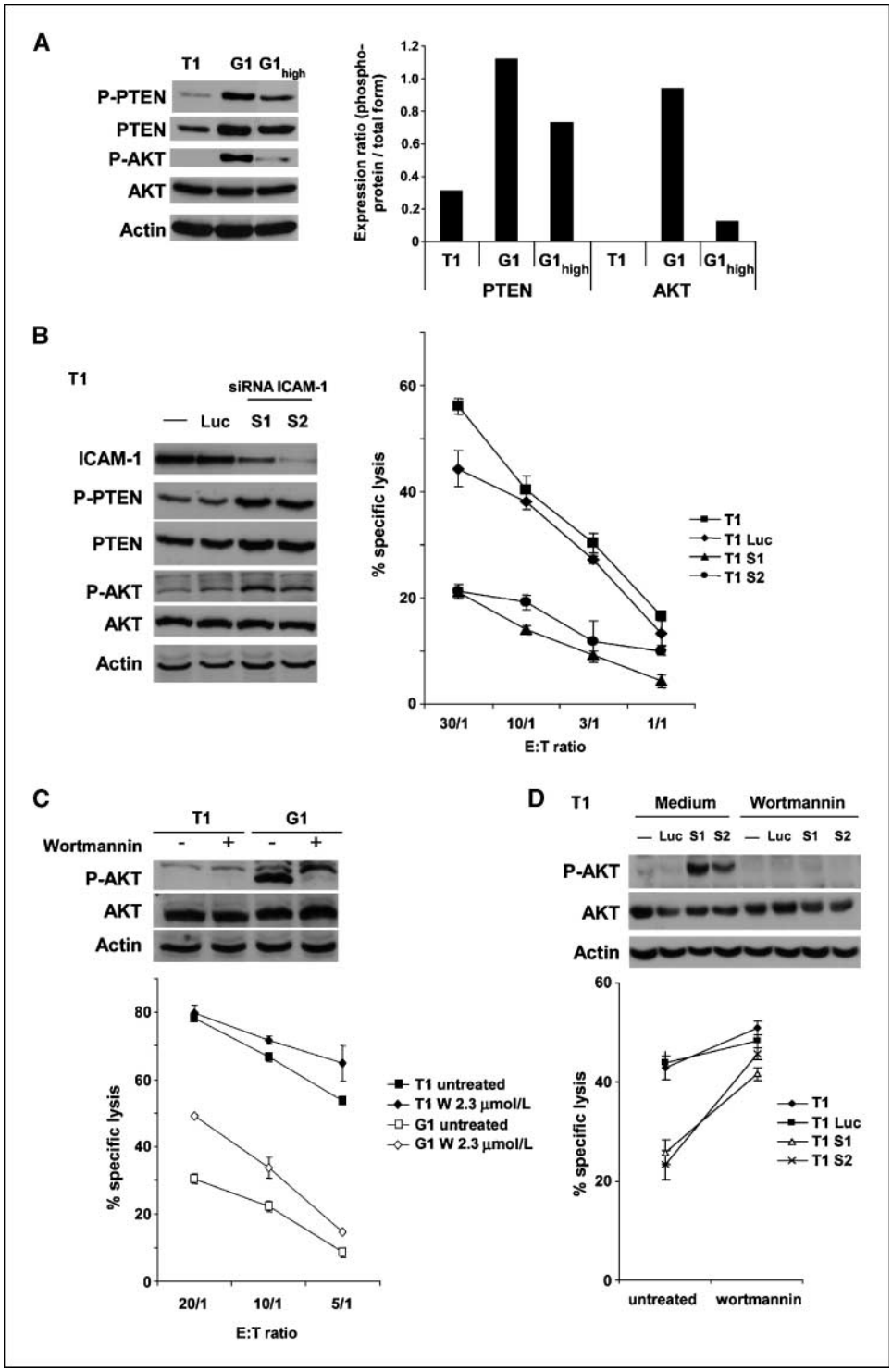


Figure 5. Effect of inhibition of PI3K/AKT pathway on G1 susceptibility to CTL–induced lysis. *A, left,* PTEN and AKT phosphorylation status in T1, G1, and ICAM-1–positive G1 tumor cells was assessed by Western blot analysis using anti-phospho-AKT (Ser^{473}), anti-AKT, anti-phospho-PTEN (Ser^{380}, Thr^{382}, Thr^{383}), and anti-PTEN mAbs. Actin was used as the protein level control. *Right,* histogram represents ratios between relative values of density of phosphorylated to total PTEN and AKT in T1 cells and ICAM-1–positive G1 and G1 cells. *B,* T1 cells were preincubated with two siRNAs (S1 or S2) against ICAM-1 or with a negative control siRNA (*Luc*) targeting luciferase for 48 h. *Left,* ICAM-1 expression and phosphorylation status and expression of PTEN and AKT were assessed by Western blot. Actin was used as the protein level control. *Right,* the lytic activity of the LT12 CTL clone toward target tumor cells was assessed in a standard 4-h chromium release assay at various E:T ratios as indicated. *C,* T1 and G1 tumor cells were pretreated or not with wortmannin (2.3 μmol/L) for 90 min before adding effectors. *Top,* phosphorylation status and expression of AKT protein was assessed by Western blot analysis; *bottom,* cytotoxicity was determined by a conventional 4-h ^{51}Cr release assay at various E:T ratios as indicated. *D,* T1 cells were preincubated with two siRNAs (S1 or S2) against ICAM-1 or with a negative control siRNA (*Luc*) targeting luciferase for 48 h. Then, these cells were pretreated or not with wortmannin (2.3 μmol/L) for 90 min before adding effectors. *Top,* phosphorylation status and expression of AKT protein were assessed by Western blot analysis; *bottom,* cytotoxicity was determined by a conventional 4-h ^{51}Cr release assay at 20:1 E:T cell ratio. All the data are representative of three independent experiments.

be sensitive to perforin-dependent cytotoxic pathways despite expression of PI-9. This unexpected finding is in support of other reports showing that some tumor cells were sensitive to cytolysis by specific T cells and cytokine-activated natural killer cells, and no difference in sensitivity was observed with respect to constitutive or induced PI-9 expression (47, 48). This suggests that PI-9 effect may be associated with the tumor target features as well as their cross-talk with the effector cells.

Accumulating evidence suggests that adhesion molecules and their signaling pathway may control the behavior of tumor cells, including cell growth, proliferation, migration, and apoptosis (49). In this respect, Anastassiou and colleagues (50) showed that the loss of ICAM-1 expression was associated with an increased risk of metastasis within the first 5 years after diagnosis of uveal melanoma. Here, we investigated the relationship between the expression of ICAM-1 and the differential susceptibility of G1

metastatic cells to CTL-induced killing and found that ICAM-1 was underexpressed in G1 metastatic cells compared with T1 primary cells. Obviously, the low expression of ICAM-1 may affect interactions with the LT12 clone as previously reported in others models (9–11). However, we show here that despite its decreased expression by G1 cells, ICAM-1 does not seem to be essential for synapse formation between CTL and target cells or for cytolytic granule polarization. Indeed, it is conceivable that the CD2/LFA-3 interaction may be sufficient to trigger CTL adhesion and degranulation toward target cells in our experimental model. Interestingly, in contrast to this lack of effect on cytotoxic synapse formation, lysis by CTL was systematically and greatly affected by varying levels of ICAM-1 expression. This suggests that the magnitude of the cytotoxic response directly correlates with levels of ICAM-1 expression and agrees with the report by Anikeeva and colleagues (51), indicating that LFA-1/ICAM-1 interaction is necessary for effective target cell lysis by the released cytotoxic granules. Taken together, these results strongly suggest that induction of ICAM-1 in resistant metastatic cells is sufficient to significantly restore the susceptibility of metastatic tumor cells to the perforin/GrB-induced apoptosis and to the CTL-mediated death.

In addition to its potential role in cell adhesion, it has been reported that the engagement of ICAM-1 leads to initiation of intracellular signal transduction, designated "outside-to-in signaling" from the cell surface (52). Recently, Langston and colleagues (22) showed that ICAM-1 regulated the expression of PTEN in mouse endothelial cells via modulation of intracellular reduced glutathione (GSH) status, suggesting a novel role in modulating vascular endothelial growth factor-A–induced angiogenesis. In the course of these studies, we identified a relationship between loss of ICAM-1 expression in G1 metastatic melanoma cells, the decrease in PTEN activity, and the subsequent activation of PI3K/AKT pathway. This differential expression of ICAM-1 and its PTEN induction was confirmed in another model of primary and metastatic melanoma (data not shown). More importantly, we showed that knockdown of ICAM-1 expression by siRNA in T1 cells was also accompanied by an increase in PTEN phosphorylation

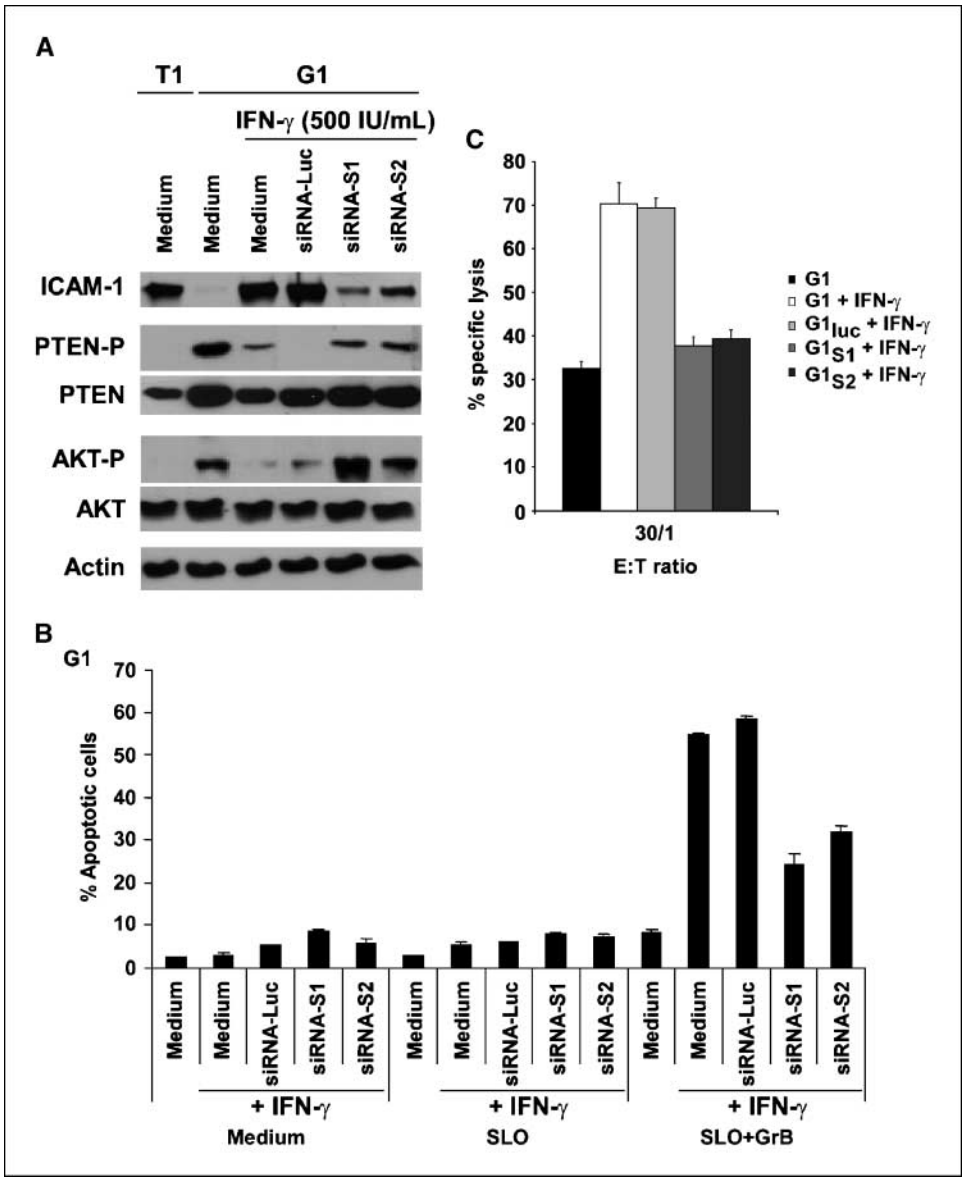


Figure 6. Effect of ICAM-1 down-regulation in IFN-γ–treated G1 cells on their susceptibility to CTL- and GrB-induced lysis. Before transfection, G1 tumor cells were cultured in medium or in the presence of IFN-γ (500 IU/mL) for 24 h. Then, G1 cells were preincubated with two siRNAs (S1 or S2) against ICAM-1 or with a negative control siRNA (*Luc*) targeting luciferase for 4 h without IFN-γ. After this incubation period, IFN-γ (500 IU/mL) was added during 48 h. *A,* ICAM-1 expression and PTEN/AKT phosphorylation status in tumor cells were assessed by Western blot analysis using anti-phospho-AKT (Ser^{473}), anti-AKT, anti-phospho-PTEN (Ser^{380}, Thr^{382}, Thr^{383}), and anti-PTEN mAbs. Actin was used as the protein level control. *B,* tumor cells were incubated with SLO (2 μg/mL) alone or in combination with recombinant GrB complex (100 nmol/L) for 90 min. Apoptosis was assessed by $Dioc_6(3)$ and propidium iodide staining. *C,* the lytic activity of LT12 clone toward tumor cells was assessed in a standard 4-h chromium release assay at 20:1 E:T ratio.

and the subsequent AKT activation. ICAM-1 cross-linking induced an increase (1.3- to 1.5-fold increase) in PTEN induction. However, it should be noted that although ICAM-1 cross-linking induced tyrosine phosphorylation, it had no effect on PTEN phosphorylation (see Supplementary Data). Nevertheless, coculture experiments of T1 cells with LT12 CTL clone did not result in PTEN induction and its phosphorylation (data not shown). This fits with the demonstration that the level of expression of PTEN was reported to affect the initiation and progression of tumors (53, 54) and that the loss of PTEN function resulted in an accumulation of PIP-3 and in AKT hyperactivation, leading to protection from various apoptotic stimuli (55). These studies also show that ICAM-1 down-regulation by siRNA in IFN-γ–treated G1 cells reverses the IFN-γ–induced higher lysis and further emphasize the existence of a relationship between ICAM-1 expression and PTEN/AKT status. In this regard, Langston and colleagues (22) have provided data indicating that constitutive ICAM-1 expression acts as an γGCL-dependent repressor of GSH level and that PTEN levels, which are sensitive to GSH status, were more expressed in ICAM-1–deficient endothelial cells than in wild-type endothelial cells. They suggested therefore that loss of ICAM-1 expression may result in an increase in PTEN activity by up-regulating protein expression without affecting the fraction of phosphorylated PTEN. In contrast, our studies in melanoma model underlined that the loss of ICAM-1 expression resulted in an increased PTEN expression with the fraction of phosphorylated PTEN suggesting a decreased PTEN activity. Although several reports indicate that ICAM-1 by its cytoplasmic tail is able to interact with actin-binding protein (α-actinin, ERM proteins), β-tubulin, glyceraldehyde-3-phosphate dehydrogenase, and PIP-2, a molecule implicated in signaling cascade and in particular a ligand of PTEN, the mechanism underlying PTEN regulation by ICAM-1 is not yet clear and will be investigated in our experimental model.

Our studies suggest that ICAM-1 is an important factor in the control of human metastatic melanoma cell susceptibility to specific lysis by CTL. It is therefore conceivable that a shift in ICAM-1 expression, which was associated with decreased PTEN activity and an activation of PI3K/AKT pathway, can be used by human metastatic melanoma cells as a strategy to promote their resistance to CTL-mediated killing.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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ICAM-1 Has a Critical Role in the Regulation of Metastatic Melanoma Tumor Susceptibility to CTL Lysis by Interfering with PI3K/AKT Pathway

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