Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors

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The concept for cellular immunotherapy of solid tumors relies heavily on the capacity of class I MHC-restricted cytotoxic T lymphocytes (CTLs) to eliminate tumor cells. However, tumors often have managed to escape from the cytolytic machinery of these effector cells. Therefore, it is very important to chart the mechanisms through which this escape can occur. Target-cell killing by CTLs involves the induction of apoptosis by two major mechanisms: through death receptors and the perforin/granzyme B (GrB) pathway. Whereas tumors previously were shown to exhibit mechanisms for blocking the death receptor pathway, we now demonstrate that they also can resist CTL-mediated killing through interference with the perforin/GrB pathway. This escape mechanism involves expression of the serine protease inhibitor PI-9/SPI-6, which inactivates the apoptotic effector molecule GrB. Expression of PI-9 was observed in a variety of human and murine tumors. Moreover, we show that, indeed, expression results in the resistance of tumor cells to CTL-mediated killing both *in vitro* and *in vivo*. Our data reveal that PI-9/SPI-6 is an important parameter determining the success of T cell-based immunotherapeutic modalities against cancer.

It is widely accepted that the induction of an efficient antitumor, cytotoxic T lymphocyte (CTL)-mediated response is achieved by presentation of the relevant tumor-associated antigen by professional antigen-presenting cells (1–4). Once primed, these CTLs can form an important barrier to the development of cancer (5). Nevertheless, in several cases, it is clear that tumors can develop properties that enable them to escape from tumor-specific CTLs *in vivo* (6, 7). Several distinct types of escape mechanisms have been described over the years (8). We and others recently provided evidence that one of these escape strategies involves the expression of antiapoptotic molecules (9, 10).

CTLs lyse their targets by means of two distinct mechanisms involving granule exocytosis and the crosslinking of so-called death receptors on the target cell (11–13). The first mechanism depends on the actions of several constituents of the secreted granules that contain the pore-forming molecule perforin together with a variety of granule-associated enzymes (such as granzymes and granulysin). Of these enzymes, granzyme B (GrB) is the main effector when dealing with target-cell apoptosis (14, 15). After secretion by the CTLs, GrB binds to the mannose-6-phosphate receptor on target cells and then enters the target cell through receptor-mediated endocytosis (16, 17). Subsequently, GrB is released from these endosomes into the cytoplasm of the target cell because of the pore-forming capacity of perforin (17, 18). Once inside the target cell, GrB cleaves several substrates, among which are Bid, inhibitor of caspase-activated DNase (ICAD), and caspase-3, -7, and -8 (19–22). In effect, the combined actions of GrB will lead to the destruction of the target cell through apoptosis. Consequently, the loss of GrB function severely hampers the capacity of CTLs to induce apoptosis in their targets (14, 15).

The second cytotoxic mechanism exerted by a CTL occurs through a subset of the tumor necrosis factor receptor superfamily (11–13) called the death receptors, which have the capacity to induce apoptosis upon oligomerization (23). Death receptor crosslinking induces a caspase cascade that will lead to the destruction of the target cell (23). Death receptor-induced apoptosis can be prevented by the antiapoptotic protein cellular-FLIP (c-FLIP; ref. 24). In agreement, c-FLIP protects cells from CTL-induced apoptosis mediated via the death receptor pathway (9, 25). c-FLIP is overexpressed in several human melanomas (26) as well as in colon and cervical carcinomas (J.P.M. and J.d.J., unpublished observations). Moreover, by using murine-tumor models, we and others have shown that overexpression of c-FLIP results in escape from CTL-mediated cytotoxicity *in vivo* by blocking death receptor-dependent cytotoxicity (9, 10). Together, these findings provide compelling evidence that c-FLIP can mediate immune evasion of tumors. It is important to note, however, that c-FLIP can prevent only death receptor-induced death but cannot block perforin/GrB-mediated cytotoxicity (9, 25).

Recent evidence indicates that protease inhibitor (PI)-9, a serine protease inhibitor (serpin), can efficiently and irreversibly inactivate GrB *in vitro* (27). Moreover, overexpression of this serpin prevents CTL-induced killing via the perforin pathway (28). As determined from Northern blot analysis, expression of PI-9 in normal somatic tissues is observed mainly in lymphoid tissue, immune-privileged sites, and T lymphocytes (27). This expression pattern suggests that PI-9 protects CTLs from destruction by their own GrB. Similar observations were reported for the murine counterpart of PI-9, SPI-6 (29). More recently, immunohistochemical analysis revealed expression in several immune-privileged sites as well (30). In addition, we recently found that expression of this serpin also protects mature dendritic cells from perforin-dependent apoptosis (31). The capacity of PI-9 to protect cells from perforin-dependent destruction makes this serpin an intriguing candidate for immune escape by tumors. This notion prompted us to analyze the expression of

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Abbreviations: CTL, cytotoxic T lymphocyte; GrB, granzyme B; c-FLIP, cellular-FLIP; PI-9, protease inhibitor-9; serpin, serine protease inhibitor; SPI-6, the murine counterpart of PI-9; SV, vesicular stomatitis virus.

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PI-9 and SPI-6 in human and murine tumors, respectively. We show here that expression of this serpin is observed in a subset of human melanomas as well as in human breast, cervical, and colon carcinomas. Furthermore, we demonstrate that SPI-6 expression in murine-tumor cells results in resistance to CTL-dependent elimination.

Materials and Methods

Tumor Tissue and Cell Lines. Human melanoma and breast carcinoma lines were cultured in DMEM supplemented with 8% (vol/vol) FCS, glutamine, penicillin, and streptomycin. Human colon and cervical carcinoma lines were cultured in Iscove's modified Dulbecco's medium (IMDM)/8% (vol/vol) FCS, glutamine, penicillin, and 2mercaptoethanol (β-ME). Human primary colon-carcinoma material was snap-frozen directly upon resection and stored at −70°C until usage. Murine-tumor lines XhoC3 (32) and AF11 (9), the chemically induced colon-carcinoma lines MC38 (33) and CMT93 (34), the lung-epithelial tumor TC-1 (35), as well as MBL-2Fas (9) and its stable transfectants were cultured in IMDM/8% (vol/vol) FCS and glutamine, penicillin, and β-ME plus 2 μg/ml puromycin [AF11, MFF, and MFF/SPI-6] or 400 μg/ml G418 (TC-1). The adenovirus E1B-specific and GagLeader-specific CTL clones were cultured as described (9).

RNA Isolation, cDNA Synthesis, and PCR. RNA from the melanoma, breast epithelial, and carcinoma lines was isolated by using RNAzol. RNA from the colon and cervix carcinoma lines was isolated by using TRIzol (GIBCO/BRL). In short, cells were resuspended in RNAzol or TRIzol and extracted with chloroform. RNA was then precipitated from the aqueous phase with ethanol and dissolved in water. Synthesis of cDNA was performed by using 2 μg of total RNA, oligo(dT) as a primer, and avian myeloblastosis virus reverse transcriptase (Promega).

PCR for murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed with GAGCCAACGGGTCATCATCT and GAGGGGCCATCCACAGTCTT as primers at an annealing temperature of 58°C and with 21, 23, or 25 cycles as indicated, yielding a 232-bp product. For human GAPDH (328 bp, 26 cycles), GGTCGGAGTCAACGGATTTG and ATGAGCCCCAGCCTTCTCCAT were used as primers and annealing was done at 61°C. SPI-6 was amplified at an annealing temperature of 42°C with CTCTGCATCATGAATACT and CCTTAAAGGTTTGGAGGA as primers and 24, 27, or 30 cycles as indicated, yielding a 336-bp product. Human PI-9 was amplified in 30 or 32 cycles with TCTGCCCTGGCCATGGTTCTCCTA and CTGGCCTTTGCTCCTCCTGGTTTA as primers and an annealing temperature of 58°C.

Western Blot Analysis. Antibody against SPI-6 was generated by the injection of recombinant denatured His-tagged full-length SPI-6 at 1 mg per rabbit. Rabbits were boosted twice, and the serum was tested subsequently against an overlapping panel of peptides spanning the complete SPI-6 protein. Positive peptides were used to purify the polyclonal serum resulting in a peptide-specific purified polyclonal rabbit serum, which showed clear specificity for the full-length protein. Cells were lysed as described (9), and proteins were separated on an SDS/10% polyacrylamide gel and blotted onto nitrocellulose (Amersham Pharmacia). Blots were blocked for 1 hr at room temperature in block buffer (PBS, 0.1% Tween-20 containing 5% milk, and 0.5% BSA). Blots were incubated with 2 μg/ml anti-SPI-6 antibody in block buffer overnight at 4°C, in horseradish peroxidase-coupled goat anti-rabbit for 1 hr, and then developed by using enhanced chemiluminescence.

***In Vitro* Translation/Association Assay.** SPI-6 was *in vitro* transcribed/translated by using a T7-based reticulate lysate reaction (Promega) and a pcDNA3-SPI-6 expression vector encoding the complete SPI-6 coding region. The resulting material was incubated for 30 min at 37°C in a buffer containing 20 mM Hepes at pH 7.5 and 100 mM NaCl, with either purified recombinant GrB, neutrophil elastase, cathepsin G (P. Hiemstra, Leiden University Medical Center, Leiden, The Netherlands), or purified murine-cytotoxic granules (J. Tschopp, University of Lausanne, Lausanne, Switzerland). Importantly, the murine granules were preincubated with 1% Triton X-100 for 5 min on ice to disrupt the membrane structure and, subsequently, diluted in incubation buffer and added to the *in vitro* translated material. As a result, the final Triton X-100 concentration in the incubation was 0.2%. After this incubation, samples were emerged in reducing sample buffer, boiled for 5 min, and separated on SDS/PAGE. The resulting gels were dried and exposed to autoradiograms.

DNA Fragmentation Assay. The DNA fragmentation assay, performed essentially as described (9), detected DNA fragmentation (36), a hallmark in the induction of apoptosis. In short, murine-tumor cells were labeled with 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine overnight, after which they were washed three times. Cells were preloaded with 1 μg/ml adenovirus E1B peptide for 15 min and then incubated for 6 hr at 37°C with an E1B-specific CTL clone at different effector-to-target cell ratios. Assays were performed with 1,000 cells (1,000–2,000 cpm) per well and in sextuplicate to minimize variation. The amount of [^{3}H]thymidine "released" by the target cell represents the amount of fragmented DNA (i.e., apoptosis) and is calculated indirectly by measuring [^{3}H]thymidine that is retained in the nuclei by using a standard harvesting protocol and the following formula: specific release = [1 − (exp. count − max count)/(medium count − max count)] × 100%. The max count represents the amount of label retained after incubation for 6 hr with 1 M HCl, which chemically degrades the DNA. To ascertain that released thymidine is not incorporated into other nuclei, assays were supplemented with unlabeled thymidine (1.5 μM).

Nicoletti Analysis of DNA Content. Cells were seeded at 0.2×10^6 cells in 0.5 ml of medium and treated with 1 μg/ml crosslinked anti-CD95 (monoclonal antibody JO-2 + 10 ng/ml protein A). After 24 hr, cells were collected and suspended in a Nicoletti buffer (0.1% sodium citrate, pH 7.4/0.1% Triton X-100/50 μg/ml propidium iodide). DNA content present in the resulting nuclei was determined on a fluorescence-activated cell sorter (FACS; Becton Dickinson). Sub-G_0/G_1 was considered apoptotic; the cellular debris was excluded from the analysis.

IFNγ Release. CTLs were seeded at 2,000 cells per 96-well plate in the presence of 5 units/ml IL-2. First, targets were loaded with 1 μg/ml E1B peptide for 30 min, and nonbound peptide was washed away. Then, targets were added to the wells at an effector-to-target cell ratio of 30:1 and left for 24 hr at 37°C. Supernatants were harvested, diluted 2-fold, and tested for the presence of IFNγ with conventional sandwich ELISA.

***In Vivo* Cytotoxicity Assay.** The *in vivo* cytotoxicity assay is based on an assay reported by Ritchie *et al.* (37). In short, MFF cells were labeled for 15 min with 10 μM 5-(and-6)-y-chloromethylbenzoyl-amino-tetramethyl rhodamine (CMTMR; Molecular Probes), which emits orange fluorescence, at 5×10^6 cells per ml and then washed three times with PBS. Labeled MFF cells and MFF/SPI-6 cells, which express high levels of enhanced green fluorescent protein, were subsequently loaded with E1B peptide (1 μg/ml) for 1 hr, after which they were washed twice with PBS/0.5% BSA. These peptide-loaded fluorescently labeled MFF and MFF/SPI-6 cells then were mixed at a 1:1 ratio and injected i.p. into nude C57BL/6 mice (6×10^6 cells per mouse). At 3 hr later, mice were either left untreated or injected i.p. with

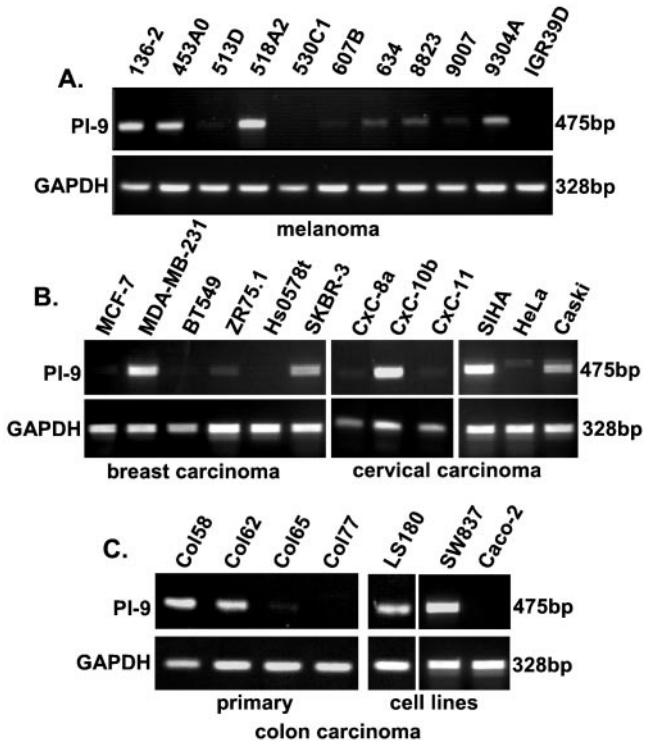

Fig. 1. PI-9 is expressed in a subset of human tumors. RNA from human melanoma (*A*), breast and cervical carcinoma (*B*), primary colon carcinoma (*C Left*), and colon carcinoma cell lines (*C Right*) is isolated, and cDNA is generated. Subsequently, a control PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*A–C Lower*) or PI-9 (*A–C Upper*) is performed.

6×10^6 E1B-specific CTL. After 24 or 48 hr, mice were killed, and the peritoneum was flushed with PBS to isolate the tumor cells. Isolated cells were analyzed on a FACSCaliber (Becton Dickinson) for fluorescence emission in fl-1 and fl-2 after gating on the tumor cells in the forward–sideward scatter plot. The ratio between orange-emitting cells (MFF) and green-emitting cells (MFF/SPI-6) was then calculated (orange-emitting cells/green-emitting cells).

Results

PI-9 Is Expressed in a Subset of Human Tumors. To analyze whether the antiapoptotic serpin PI-9 is expressed in human tumors, we screened a panel of human tumor lines for expression of this gene. As is clear from Fig. 1, expression of PI-9 mRNA was detected in a panel of melanoma lines that were generated by direct culturing of resected tumor material (Fig. 1*A*). Expression of PI-9 also was observed in breast and cervical carcinoma lines (Fig. 1*B*). Importantly, expression was observed in only a subset of the tumor lines tested, excluding a general effect of tumor development or *in vitro* passage of tumor lines. A similar pattern of PI-9 expression also was detected in a panel of primary surgical specimens of colon carcinomas (Fig. 1*C Left*). Accordingly, two of the three human colon-carcinoma cell lines tested were found to express PI-9 (Fig. 1*C Right*). Taken together, our data suggest that the expression of PI-9 has a role in the protection of tumor cells against elimination by CTL.

The Murine Serpin SPI-6 Is Expressed in Murine Tumors. To delineate further the role of the GrB-inhibitory serpin PI-9 in the immune escape of tumors, we examined the consequence of expression of the murine homologue of this GrB-inhibiting serpin, SPI-6, in murine-tumor models. Like PI-9, SPI-6 binds irreversibly to GrB, which can be visualized on SDS/PAGE as a ''shifted'' complex (Fig. 2*A*; ref. 29). This complex not only was observed when purified GrB was used but also could be detected with purified granules from murine CTLs (Fig. 2*A*). This observation indicates that SPI-6 can target the GrB present in these granules, and that it can negatively inhibit the activity of GrB in a similar fashion as PI-9.

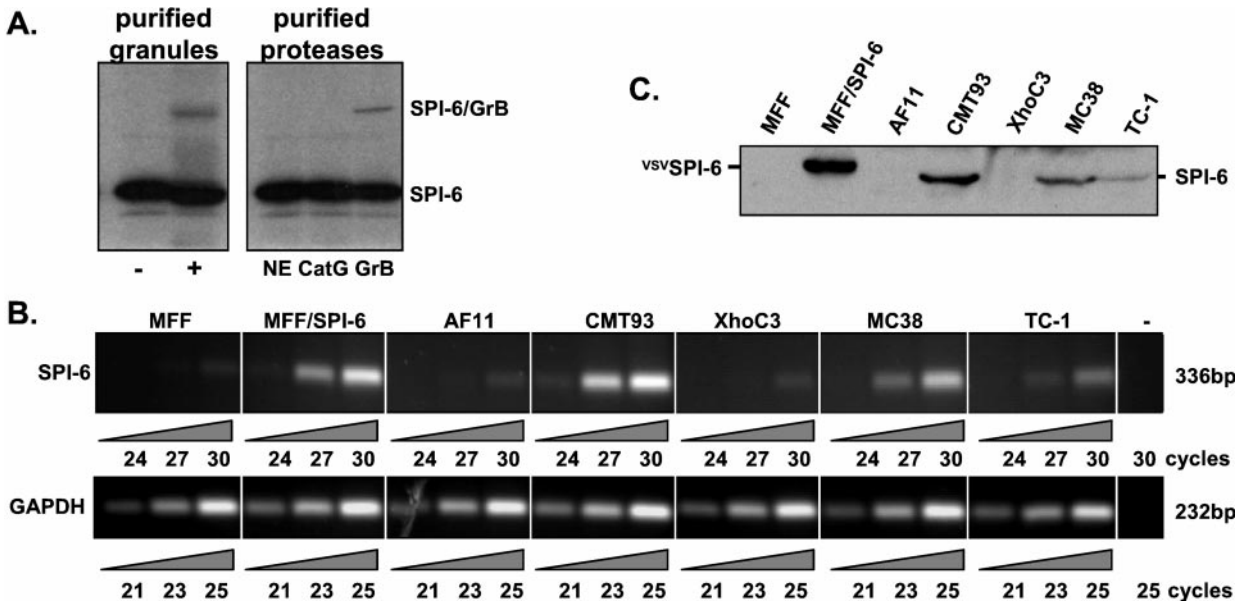

Fig. 2. SPI-6 binds GrB and is expressed in a subset of murine tumors. (*A*) *In vitro* translated [^{35}S]methionine-labeled SPI-6 was incubated in the absence (−) or presence (+) of purified lysed granules (*Left*) or in the presence of neutrophil elastase (NE), cathepsin G (CatG), or GrB (*Right*). Complex formation was detected with the use of a mobility shift in an SDS/PAGE gel. (*B*) RNA isolated from different murine-tumor lines was used for reverse transcription–PCR. (*Bottom*) GAPDH PCR for 21, 23, and 25 cycles. (*Upper*) SPI-6 PCR for 24, 27, and 30 cycles. (*C*) Western blot analysis of lysates from 0.5×10^6 cells using a peptide-purified rabbit polyclonal antiserum against SPI-6. Please note that the SPI-6 in MFF/SPI-6 migrates at a different mobility because it is tagged with a short sequence derived from the vesicular stomatitis virus (VSV) glycoprotein at the N terminus.

Next, we analyzed the expression of SPI-6 mRNA in a panel of murine C57BL/6-derived tumor lines. This analysis revealed that the expression of SPI-6 differed greatly among these tumors (Fig. 2*B*). Strong expression was detected in the chemically induced colon-carcinoma lines MC38 and CMT93 (33, 34) as well as in the lung–epithelial tumor TC-1, which is an HPV-16 E6/E7 plus mtRas-transformed tumor line (ref. 35; Fig. 2*B*). Expression was most prominent in CMT93, which contains about 50- to 100-fold more SPI-6 mRNA as compared with XhoC3, AF11, and MFF (Fig. 2*B*). For functional analysis, we generated a transfectant of the MFF tumor line that efficiently expresses SPI-6 [tagged with a short sequence derived from the vesicular stomatitis virus (VSV) glycoprotein VSV at the N terminus] under the control of a retroviral long terminal repeat promoter. This MFF/SPI-6 line expresses SPI-6 mRNA at levels comparable to those of the CMT93 line (Fig. 2*B*).

To determine whether the differences at the mRNA level are observed at the protein level as well, we generated an antibody against SPI-6. To this end, rabbits were immunized with His-tagged full-length SPI-6, and the resulting serum was purified with peptides derived from SPI-6. With the use of this serum, we were able to analyze the expression of SPI-6 at the protein level in this set of murine tumors. This analysis revealed that the SPI-6 mRNA expression observed was reflected by expression at the protein level (Fig. 2*C*).

SPI-6 Expression Correlates with CTL Resistance. To analyze directly whether the expression of SPI-6 regulates CTL sensitivity, we tested the capacity of tumor-specific CTLs to kill these cells *in vitro*. The CTL clone used recognizes an adenoviral type 5 E1B-derived epitope in the context of H-2D^b. This MHC class I molecule is expressed on all tumor cells in this panel (not shown). Accordingly, exogenous loading of these tumors with the relevant peptide epitope and incubation with the E1B-specific CTL resulted in CTL activation by all tumor lines, as measured by IFNγ production (Fig. 3*A*).

Because all tumors were recognized by the E1B-specific CTL when loaded with the relevant peptide, we analyzed the capacity of these CTLs to induce tumor-cell apoptosis in a 6-hr DNA fragmentation assay (36). This assay measures the capacity of a CTL to induce apoptosis in its target cell and is considered to reflect more accurately *in vivo* CTL-mediated killing than chromium-release assays (16). By using this assay, we could show that XhoC3, MFF, and AF11 were highly sensitive to the actions of the CTL (Fig. 3*C*). All tumors in this panel were found to be equally resistant to tumor necrosis factor (not shown) and CD95-induced apoptosis (Fig. 3*B*); this fact indicates that CTL-induced killing of these tumors (Fig. 3*C*) is mediated via the perforin/GrB pathway (9). In contrast to the CTL-sensitive phenotype of these three tumors, we found, at the same time, that CMT93 and TC-1 are fully resistant to the actions of the E1B-specific CTL. So, within one assay, we found that E1B-specific CTLs are capable of inducing apoptosis in SPI-6-negative targets, whereas these CTLs are unable to induce apoptosis in SPI-6-expressing targets. Only the colon carcinoma MC38 shows some residual lysis (Fig. 3*D*) despite expression of SPI-6 (Fig. 2 *B* and *C*). This phenomenon is most likely caused by the relatively high MHC class I expression (not shown) and, consequently, high CTL activation achieved by this tumor line (Fig. 3*A*). Thus, MC38 is subject to more intensive CTL attack, which apparently cannot be completely neutralized by SPI-6.

Expression of SPI-6 Is Causally Related to Tumor-Cell Resistance. Although the expression of SPI-6 shows a striking correlation with the protection against CTLs, no causal connection can be deduced from these results. To analyze directly whether the expression of SPI-6 is sufficient to protect cells from perforin-dependent apoptosis, we made use of the SPI-6 transfectant of

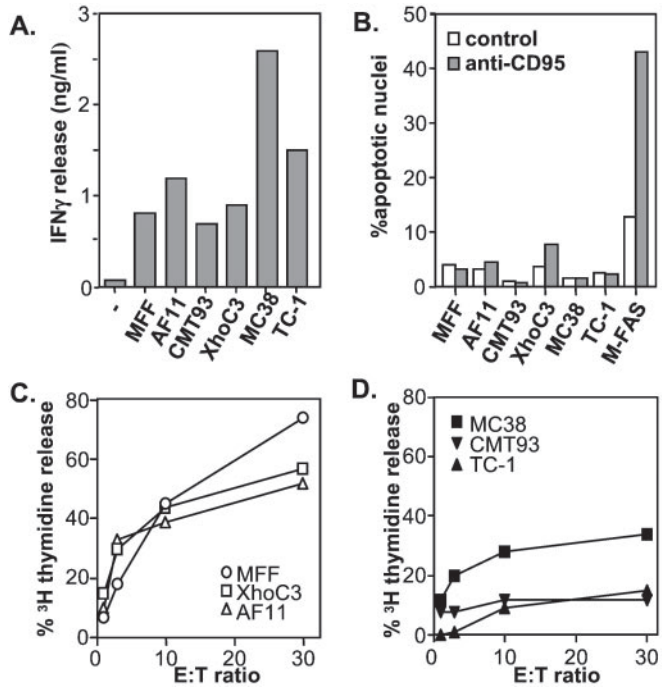

Fig. 3. SPI-6 expression is inversely correlated with lysis by CTLs. (*A*) Cells were incubated with the E1B-specific CTL clone in the presence of the relevant epitope; after 24 hr, the medium was analyzed for IFNγ secreted by the activated CTLs. (*B*) Cells were treated for 24 hr with crosslinked anti-CD95 and analyzed for DNA fragmentation with the Nicoletti assay (see *Materials and Methods*). (*C* and *D*) Cells were labeled with [^{3}H]thymidine overnight and incubated with an E1B-specific CTL clone in the presence of the relevant epitope. After 6 hr, the remaining label was determined and served as a measure for CTL-induced DNA fragmentation (apoptosis). Note that *C* and *D* represent one assay with the same CTL population. E:T ratio, effector-to-target ration.

the MFF tumor line. MFF is a Moloney virus-induced T cell lymphoma that expresses high levels of the death receptor CD95 (Fas/APO-1) but is resistant to CD95-induced apoptosis (Fig. 3*B*) caused by coexpression of the antiapoptotic protein c-FLIP (9). In effect, CTL-mediated apoptosis of MFF occurs only via the perforin/GrB pathway (9). Therefore, this cell line is representative of the murine-tumor panel tested here (Fig. 3*B*) and is a highly proper system for analysis of GrB-dependent apoptosis in the absence of a functional death receptor pathway. In addition, expression of SPI-6 was low or undetectable on both the mRNA and protein levels in MFF (Fig. 2 *B* and *C*). Therefore, this tumor line allowed us to analyze the effect of SPI-6 expression on the perforin/GrB pathway.

Incubation of MFF cells with CTLs resulted in the effective induction of apoptosis (Fig. 3*C* and Fig. 4*A*). As expected, killing is completely perforin-dependent, as it is prevented by preincubation of the CTLs with concanamycin A (Fig. 4*A*), a substance that results in the degradation of perforin (9, 38). Next, we overexpressed SPI-6 in these MFF cells and analyzed whether the expression of this serpin is a decisive factor in the sensitivity of tumor cells to the perforin pathway, which was suggested by the tumor panel tested (Fig. 3). Indeed, this MFF/SPI-6 cell is fully resistant to CTL-induced apoptosis (Fig. 4*A*), indicating that SPI-6 is a very effective inhibitor of the perforin/GrB pathway.

Importantly, the SPI-6 expression levels observed in these MFF/SPI-6 cells were comparable to what was detected in the CMT93 tumor line (Fig. 2). Therefore, we conclude that the expression of SPI-6 in these death receptor-resistant murine

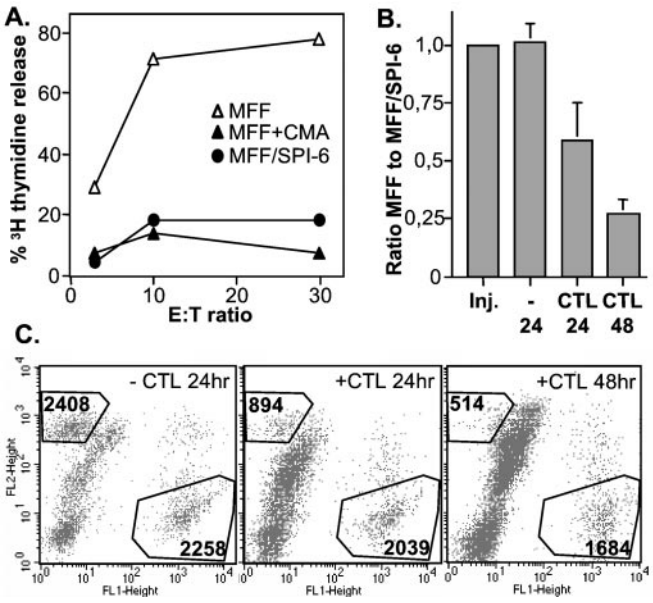

Fig. 4. SPI-6 prevents CTL-induced killing *in vitro* and *in vivo*. (*A*) Cells were labeled with [³H]thymidine overnight and incubated with the Moloney murine leukemia virus antigen-specific CTL clone. DNA fragmentation of MFF (△) or MFF/SPI-6 (●) was determined 6 hr later. To determine the contribution of the perforin pathway, CTLs were preincubated with concanamycin A before the addition of the MFF cells (▲). (*B*) MFF cells were *in vitro* labeled with CMTMR (orange fluorescence) and loaded with E1B peptide. MFF/SPI-6 cells, which enhanced green fluorescent protein, were loaded similarly with E1B peptide. MFF and MFF/SPI-6 cells were mixed subsequently at a 1:1 ratio and injected i.p. into nude mice. Mice were then injected i.p. with E1B-specific CTLs or were left untreated. At 24 or 48 hr later, tumor cells were isolated, and the MFF to MFF/SPI-6 ratio was determined from five mice per timepoint (standard deviation is given). The first bar is the ratio of cells that were injected (1:1). (*C*) One representative FACScan profile is shown per group.

tumors is a critical determinant of the sensitivity of these cells for the perforin/GrB-dependent apoptosis *in vitro*.

Subsequently, we set out to determine the relevance of these findings for immune escape of tumor cells *in vivo*. To this end, CTL-sensitive MFF cells were fluorescently labeled (orange) and injected into nude mice (1:1 ratio) together with MFF/SPI-6 (green fluorescence). Both tumor cells can be recovered easily from the mouse 24 hr later and, importantly, at a 1:1 ratio (Fig. 4 *B* and *C*). This fact indicates that the cells show comparable behavior and survival after injection. However, when tumor-specific CTLs were injected into the mouse, we found a selective loss of the MFF cells after 24 hr, as compared with the MFF/SPI-6 cells (Fig. 4 *B* and *C*). This loss was even more pronounced after 48 hr (1:4 ratio). Importantly, despite the clear decrease in MFF cells, the MFF-SPI-6 cells were recovered from the mice after 48 hr (Fig. 4*C*). Taken together, these results indicate that expression of SPI-6 in these MFF/SPI-6 cells permits escape from killing by the CTLs *in vivo*. Therefore, we conclude that expression of this serpin in both human and murine tumors represents a previously unrecognized mechanism by which tumors escape from immune surveillance.

Discussion

CTLs constitute a crucial barrier in the development of tumors, and escape of this surveillance is, therefore, a common theme in tumorigenesis. Several different escape mechanisms have been described (8), ranging from MHC class I down-regulation to the expression of immune-modulatory cytokines. We and others have provided evidence that tumors use the death receptor inhibitor c-FLIP as a way to escape from the cytotoxic actions of a CTL (9, 10, 26). Here, we show that tumors can employ a distinct antiapoptotic mechanism to prevent CTL-dependent cytotoxicity via the perforin pathway. This mechanism involves PI-9/SPI-6, a serpin that specifically inactivates GrB and, thereby, prevents perforin-dependent apoptosis. Importantly, we demonstrated that expression of SPI-6 is causally related to the resistance of tumor cells, because overexpression of this serpin in an otherwise sensitive tumor line renders this line resistant to CTL-induced killing *in vitro* as well as *in vivo* (Fig. 4).

It is important to note that we have used DNA fragmentation, a hallmark of apoptosis (39), in our *in vitro* experiments, because it was recently demonstrated that this method more closely represents the *in vivo* situation, as compared with the classical chromium-release assay. In this study, it was shown that cells lacking the receptor for GrB are protected from GrB-induced death and cannot be lysed *in vivo* by infiltrating CTLs (16). This fact indicates that *in vivo* CTL-induced cytotoxicity depends on the receptor for GrB. In agreement, receptor-positive cells were readily eradicated by the CTL *in vivo*. Despite their *in vivo* resistance, the GrB receptor-deficient cells can be lysed easily by CTLs *in vitro* when tested in the chromium-release assay, whereas CTL-induced apoptosis as measured by DNA fragmentation was not observed (16). DNA fragmentation was observed, however, when the GrB-receptor-expressing cells were used as targets. Taking these findings together, this report demonstrates that the cytotoxic effects observed in the chromium-release assay are not translatable directly to the *in vivo* situation, whereas DNA fragmentation is the assay of choice when sensitivity to CTL-mediated killing is to be extrapolated to an *in vivo* situation. In agreement with these findings, we have not been able to detect a clear-cut inhibition of CTL-induced chromium release by SPI-6 (not shown). Although others have reported prevention of LAK cell-induced chromium release (28), in our hands, the inhibition of CTL-induced release is only marginal. Despite this *in vitro* sensitivity in the chromium-release assay, we do find that SPI-6 protects against CTL-induced apoptosis, and, more importantly, we provide direct evidence that this protection is relevant for the survival of tumor cells in the face of activated CTL *in vivo*. Therefore, we conclude that PI-9/SPI-6 expression represents a potent mechanism for tumors to escape from the perforin/GrB pathway.

Previous reports have highlighted the role of the perforin pathway in tumor control. For example, perforin-deficient mice are less capable of rejecting transplanted tumor cells (40). In addition, the incidence of spontaneous lymphomas is dramatically increased in mice that are deficient in both perforin and p53 as compared with mice deficient in p53 alone (41). In view of these findings, it is conceivable that the inhibition of GrB-dependent killing by expression of SPI-6/PI-9 by the tumor will greatly hamper the attack by CTLs. We now show that death receptor-resistant tumor cells expressing high levels of SPI-6 can, indeed, resist killing *in vivo* (Fig. 4). However, by using GrA/GrB-deficient mice, others have shown that these granzymes can be dispensable for tumor eradication (42). One explanation for this apparent discrepancy is that mice may compensate for the genetic loss of GrB by an increased expression of other granzymes. Alternatively, one could envision that the absence of GrB-dependent killing is not the sole factor determining survival, and that other pathways need to be blocked simultaneously to cause escape of CTL-induced killing. In this context, it is important to note that PI-9/SPI-6 can prevent perforin-dependent apoptosis but are ineffective against death receptor-dependent cytotoxicity (28). Even so, all tumors in the tumor panel analyzed by us are inert to death receptor-dependent apoptosis (Fig. 3*B*). We have found that this condition is essential for PI-9/SPI-6 to protect cells from CTLs, as expression of SPI-6 in a tumor that is CD95-sensitive does not affect the overall *in vitro* CTL-sensitivity (not shown). Notably, resistance

to death receptor-mediated death is a very common feature of tumors. It is achieved not only by expression of c-FLIP (9, 10, 26), but also by a variety of other mechanisms such as death receptor down-regulation or mutation (43, 44), caspase-8 mutation or methylation (45, 46), or decoy receptor secretion (47). Apparently, tumors have a plethora of mechanisms at their disposal to prevent death receptor-dependent apoptosis at different stages of the apoptotic signaling cascade. When combined with SPI-6 expression, inhibition of death receptor-induced apoptosis will protect tumor cells from CTLs completely—as is exemplified by the c-FLIP/SPI-6 double transfectant used in this study (Fig. 4)—and will, therefore, allow a tumor to escape from T cell-dependent immunity.

Expression of PI-9/SPI-6 was not observed in all tumors tested, indicating that it is not a general feature of tumors. Recently, endogenous expression of PI-9 was reported in dendritic cells (30, 31), lymphocytes, and specific cell types in immune-privileged sites (30). PI-9 levels are not detectable in normal melanocytes, breast or cervical epithelium, or colon (30). These observations strengthen our conclusion that the expression of PI-9/SPI-6 in a number of the tumors tested is, indeed, tumor-specific and is a feature that can endow the tumor with a CTL-resistant phenotype. In conclusion, expression of PI-9/SPI-6 is of crucial importance in the escape of tumors from a CTL response and is, therefore, a parameter that influences the feasibility of CTL-mediated immunotherapy of cancer.

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