Antigen Presented by Tumors In vivo Determines the Nature of CD8+ T-Cell Cytotoxicity

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Abstract

The biological relevance of the perforin and Fas ligand (FasL) cytolytic pathways of CD8+ T lymphocytes (CTL) for cancer immunotherapy is controversial. We investigated the importance of these pathways in a murine renal cell carcinoma expressing influenza viral hemagglutinin as a defined surrogate antigen (Renca-HA). Following Renca-HA injection, all FasL-dysfunctional FasLgd/gd mice (n = 54) died from Renca-HA tumors by day 62. By contrast, perforin−/− (51%; n = 45) and FasLpr/p (55%; n = 51) mice remained tumor-free at day 360. Blocking FasL in vivo inhibited tumor rejection in these mice. Moreover, established Renca-HA tumors were cleared more efficiently by adoptively transferred HA518–526-specific T-cell receptor–transgenic CTL using FasL rather than perforin. Strikingly, a range of mouse tumors cells presenting low concentrations of immunogenic peptide were all preferentially lysed by the FasL but not the Pfp-mediated effector pathway of CTL, whereas at higher peptide concentrations, the preference in effector pathway usage by CTL was lost. Interestingly, a number of human renal cancer lines were also susceptible to FasL-mediated cytotoxicity. Therefore, the FasL cytolytic pathway may be particularly important for eradicating Fas-sensitive tumors presenting low levels of MHC class I–associated antigens following adoptive T-cell therapy.

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Introduction

CTLs form the basis of adoptive T-cell therapy of cancer. Despite various optimizations of adoptive T-cell therapy (1), the majority of patients still fail to show objective cancer regression even in melanoma (1) and renal cell carcinoma (2), the two cancers most responsive to immunotherapy to date. To further improve T-cell immunotherapy against cancer, we thus need to understand the molecular basis of tumor eradication by CTL in vivo.

Two direct mechanisms of CTL cytotoxicity have been defined in vitro (3). One involves the perforin (Pfp)-dependent exocytosis of lytic granules (4, 5), whereas the second mechanism uses various apoptosis-inducing death ligands of the tumor necrosis factor (TNF) superfamily (6). Decreased rejection of induced sarcomas and papillomas (7, 8) or spontaneous lymphomas (9) in Pfp−/− mice suggested that Pfp may be important for CTL cytotoxicity in vivo. Nonetheless, the adoptive transfer of Pfp-deficient CTL to tumor-bearing mice showed substantial antitumor therapeutic efficacy (10–12). From these studies, no definite conclusions, however, could be drawn concerning the molecular basis of tumor elimination following T-cell transfer, the importance of any alternative cytotoxic pathways in Pfp-replete mice, functional dominance or redundancy of direct cytotoxic pathways, and the role of the biological characteristics of the tumor cells in determining the nature of the CTL effector functions.

Materials and Methods

Mice. All BALB/c mice (7–12 wk) were bred at the National Cancer Institute (NCI)-Frederick. NCI-Frederick is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and follows the Public Health Service Policy for the care and use of laboratory animals under pathogen-free conditions. Cln−4 mice (courtesy Linda A. Sherman, The Scripps Research Institute, La Jolla, CA) were established on C57BL/6 (B6) and Pfp−/− (13, 14) background. B6 and Pfp−/− mice were used at B6 and Pfp−/− wild-type or Pfp−/−/− background.

Cell lines. The Renca-HA line (courtesy Hyam I. Levitsky, John Hopkins University, Baltimore, MD; ref. 14), Renca (BHW, NCI-Frederick), 4T1 (courtesy Suzanne Ostrand-Rosenberg, University of Maryland Baltimore County, Baltimore, MD), C-26 and A-20 (American Type Culture Collection), and ACHN, TK-10, and UO-31 (DTP Molecular Targets Program, NCI-Frederick) were maintained in FCS-supplemented standard RPMI 1640 culture medium.

Tumor monitoring. Tumor size following s.c. injection of Renca-HA cells was monitored every week, and is represented as multiplication of the two perpendicular diameters. Tumor metastatic nodules following i.v. injection with Renca-HA cells were counted on the surgically removed lungs under a dissection microscope.
Flow cytometry. Cells following FcγR blocking were stained for surface immunofluorescence staining with various antibodies or control isotypes (BD Biosciences or eBioscience, Inc.). Cells were stimulated for 4 h with 200 ng/mL ionomycin plus 10 ng/mL phorbol 12-myristate 13-acetate (Sigma) in the presence of GolgiStop protein transport inhibitor (BD Biosciences) for IFN-γ intracellular staining, or in the presence of matrix metalloproteinase inhibitor BB94 (10 μM/l; IIV) for FasL detection. For measuring effector cell degranulation, CTL were incubated with target cells for 6 h in the presence of monensin (15 μg/mL; BD Biosciences) for IFN-γ (Sigma) in the presence of GolgiStop protein transport inhibitor (BD Biosciences) or an irrelevant nucleoprotein (TYQTRALV) peptide. On day 3, cells were washed and injected i.v. in tumor-bearing recombination-activating gene deficient (RAG2−/−) mice. Strikingly, 55% (28 of 51) of Fas−/− mice had lymphoproliferative disorders, which showed similar immunologic disorders (17).

Statistics. Comparisons of mean values between the groups were analyzed using GraphPad Instat software (GraphPad Prism). Statistical significance of the differences was analyzed by using unpaired Student’s t test or by ANOVA. Comparisons of survival curves estimated by Kaplan-Meier plots using GraphPad Prism were performed by the log-rank (Mantel-Cox) test. All statistical tests were two-sided; P values of <0.05 were considered statistically significant.

Results

Antitumor role of FasL is crucial in vivo. To investigate the roles of Pfp, Fasl, and Fas in the host’s capacity to reject tumors, Renca-HA tumor cells were injected in BALB/c WT, severe combined immunodeficient (SCID), Pfp−/−, Fasl−/−/−, and Fas−/−/− mice. Following Renca-HA s.c. injection, solid tumors rapidly grew in 100% of SCID and Fasl−/−/− mice. In WT, Pfp−/−, and Fas−/−/− mice, tumors grew much more slowly and did not develop in all animals (Supplementary Fig. S1). Concerning long-term survival, all SCID mice (n = 43) died with large Renca-HA tumors with a median survival of 62 days. By contrast, 51% of WT mice (28 of 55) were tumor-free at day 360 (Fig. 1A), most likely due to an adaptive immune response against HA. In Pfp−/− mice, 51% (23 of 45) of Pfp−/− mice were tumor-free at day 360 similar to WT mice. Furthermore, in Fasl−/−/− mice (n = 54), which lacked functional Fasl protein (17), all mice died with large tumors with a median survival of 62 days. Because Fastl−/−/− mice have lymphoproliferative disorders, we compared Renca-HA growth in Fastl−/−/− mice with growth in Fastl−/−/− mice (defective Fast but functional Fasl) that show similar immunologic disorders (17). Strikingly, 55% (28 of 51) of Fastl−/−/− mice were tumor-free at day 360 (Fig. 1A). Therefore, WT, Pfp−/−, and Fas−/−/− mice resisted...
Renca-HA growth, resulting in long term survival, whereas SCID or FasL^gld/gld^ mice rapidly succumbed to tumor.

Similarly, following Renca-HA (0.5 \times 10^6) i.v. injection, WT, Pfp^-/-, and Fas^lpr/lpr^ mice formed significantly fewer lung metastases compared with SCID or FasL^gld/gld^ mice (Fig. 1B). The requirement of FasL for Renca-HA rejection was further confirmed when administration of a FasL-blocking mAb but not its isotype control in WT, Pfp^-/-, and Fas^lpr/lpr^ mice abrogated the rejection of Renca-HA lung metastases (Fig. 1B). Thus, the expression of functional FasL in the host seemed crucial for resisting the development of Renca-HA solid tumors.

**Activation of CTL in tumor-bearing mice.** To dissect the mechanisms underlying the preferential effector pathway usage in tumor rejection, we compared tumor metastases in the lungs following Renca-HA (3 \times 10^6) i.v. injection in HA-specific TCR Tg^Pfp^-/- and Tg^gld/gld^ mice (Fig. 1B). Reduced pulmonary metastases were found in all Tg mice compared with WT mice (Fig. 2A). Moreover, Tg^Pfp^-/- mice exhibited significantly fewer metastases compared with Tg^gld/gld^ mice, suggesting that HA-specific CTL expressing FasL were more efficient at causing Renca-HA rejection.

We then analyzed the activation of HA-specific CTL in tumor-bearing mice. We injected Renca-HA cells s.c. (5 \times 10^6) in WT, Tg^Pfp^-/-, and Tg^gld/gld^ mice, and evaluated the number of cells in tumor-draining LN (DLN) and contralateral LN (CLN) on day 4. The numbers of cells in the DLN of Renca-HA–bearing mice were significantly increased compared with the CLN in all of these mice. As expected, no differences were seen in nontumor-bearing mice (Fig. 2B).

The gated CD8^+ cells in the DLN of tumor-bearing WT, Tg, Tg^Pfp^-/-, and Tg^gld/gld^ mice analyzed on day 7 after tumor injection showed an up-regulated expression of CD25 and CD44 as well as a capacity to produce high amounts of IFN-\(\gamma\) (Fig. 2C). Moreover, the DLN of Tg, Tg^Pfp^-/-, and Tg^gld/gld^ mice showed a similar percentage of HA_{518-526}–specific K^d:HA-tetramer^+ cells with similar levels of T-cell activation. Thus, decreased efficiency of Tg^gld/gld^ mice to eliminate tumor cannot be attributed to deficiency in activation of HA-reactive CTL.

**FasL effector pathway is 100-fold more efficient than Pfp in vivo.** To more systematically compare the efficiency of Pfp-dependent and FasL-mediated cytolysis, we adoptively transferred a range (0.01–1 \times 10^6) of activated Tg^Pfp^-/- or Tg^gld/gld^ CTL into RAG2^-/- mice that lack endogenous T, B, and NKT cells and had been injected with Renca-HA cells (2 \times 10^6) i.v. 7 (Fig. 3A) or 10 d earlier (data not shown). Transfer of high numbers of CTL from
Tg\(^{Pfp-/-}\) or Tg\(^{gld/gld}\) mice regressed these established lung metastases without any overt toxicity. However, Tg\(^{gld/gld}\) CTL were far less efficient at promoting tumor regression when lower numbers of CTL (0.01 \(\times 10^6\)) were transferred (Fig. 3A). In addition, the therapeutic benefit following transfer of Tg or Tg\(^{Pfp-/-}\) CTL was abolished by administration of FasL-blocking antibody mAb (Fig. 3D). Activated Tg CTL did express increased granzyme B, Pfp and FasL (Fig. 3D).

Thus, Tg\(^{Pfp-/-}\) CTL that use FasL were 100-fold more efficient on a per cell basis at promoting rejection of the Renca-HA tumor \textit{in vivo} than Tg\(^{gld/gld}\) CTL, despite the fact that the transferred Tg, Tg\(^{Pfp-/-}\), or Tg\(^{gld/gld}\) cells showed similar proportions of K\(^{+}\)-HA-Tet\(^+\) CD8\(^+\) cells (Supplementary Fig. S2), produced equivalent amounts of IFN-\(\gamma\) (Fig. 3D), and trafficked to the site of tumor pulmonary metastases with similar efficacy as shown by the CD8 immunohistochemistry of lung sections from tumor-bearing mice (Fig. 3C). In addition, staining of lungs with H&E revealed punctate infiltration of lymphoid cells into distinct areas of lungs where tumor cells were present (data not shown). These results show that FasL, cytolytic effector mechanism is critical in CTL-mediated regression of Renca-HA tumors.

**Figure 3.** FasL effector pathway is more efficient than Pfp \textit{in vivo}. Lung metastases counts on day 14 following Renca-HA (2 \(\times 10^6\) i.v.) injection in RAG\(^{-/-}\) mice (A) that were transferred with the indicated numbers of the activated Tg\(^{Pfp-/-}\) and Tg\(^{gld/gld}\) LN CTL i.v. on day 7; *, \(P = 0.004\); **, \(P < 0.0001\) (ANOVA, two-sided) for Pfp\(^{-/-}\) (n = 10) compared with the corresponding gld (n = 10) group; (B) that were administered with FasL-neutralizing MFL4 or its isotype IgG on day 0, 2, and 5 after transfer of the activated Tg and Tg\(^{Pfp-/-}\) CTL (0.1 \(\times 10^6\)); *, \(P < 0.0001\) (ANOVA, two-sided) for MFL4-treated (n = 9) compared with the corresponding control IgG-treated (n = 9) group. C, the activated Tg\(^{Pfp-/-}\) and Tg\(^{gld/gld}\) LN CTL (5 \(\times 10^6\) i.v.) were transferred i.v. in RAG\(^{-/-}\) mice bearing 10-day–established Renca-HA tumor metastases. Representative sections of lungs and WT mouse control thymus harvested a day after CTL transfer and stained with CD8 \(\alpha\) Ab. Bar, 25 \(\mu\)m, all images; +ve control, positive control. Data representative of two independent experiments. D, bar graphs, IFN-\(\gamma\) secretion in the day 4 supernatants from the HA-stimulated Tg, Tg\(^{Pfp-/-}\), and Tg\(^{gld/gld}\) LN CTL cultures. Columns, means of triplicate samples in a representative of two independent experiments; bars, SD. Histograms represent the % expression of granzyme B, Pfp, and FasL on gated CD8\(^{+}\) TCR V\(_8\).1\(^+\) cells in the HA-stimulated Tg LN CTL compared with naive Tg LN T cells, with numbers depicting the mean fluorescence intensities for naive (red) and activated (green) Tg T cells.
Renal cancer cells express Fas in vivo. Renca-HA cells isolated from the solid tumors in Fas<sup>br/br</sup> or Fas<sup>gld/gld</sup> mice inoculated with a high number of Renca-HA cells (5 x 10<sup>6</sup>) expressed Fas (Supplementary Fig. S3). This was confirmed by immunohistochemistry of tumor sections from Fas<sup>br/br</sup> or Fas<sup>gld/gld</sup> mice (Fig. 4A). Moreover, 92% of HA-gated tumor cells from established solid tumor in RAG2<sup>/−/−</sup> mice expressed MHC class I, of which 66% expressed Fas (Fig. 4B). Fas surface expression on MHC class I+ tumor cells ex vivo was significantly higher than Renca-HA cells grown in vitro. Treatment of Renca-HA or Renca cells with IFNγ and TNFα up-regulated their expression of Fas (Supplementary Fig. S3; Fig. 4C). We also examined whether Fas engagement on Renca-HA cells triggered productive apoptotic signaling. Engagement of Fas on Renca-HA cells by FasL expressed on CTL triggered tumor cell apoptosis as it was blocked by a pan-caspase inhibitor zVAD-FMK (Supplementary Fig. S4A).

Nonredundant roles of FasL and Pfp effector pathways. To investigate why Fasl pathway was dominant in CTL-mediated rejection of Renca-HA tumors in vivo, we evaluated the cytolytic activity of stimulated Tg<sup>/−/−</sup>, Tg<sup>br/br</sup>, and Tg<sup>gld/gld</sup> CTL against the Renca-HA transfectants and parental Renca tumor cells pulsed with increasing concentrations of exogenous HA peptide. Renca cells loaded with >10 nmol/L concentration of HA peptide were lysed efficiently by all three types of effector CTLs (Fig. 5A). However, at 10<sup>−10</sup> to 10<sup>−12</sup> mol/L concentrations of HA, only Tg or Tg<sup>br/br</sup> CTL expressing Fasl killed tumor cells, whereas Tg<sup>gld/gld</sup> effectors using Pfp did not. In the same assay, lysis of endogenous HA-expressing Renca-HA cells by Tg or Tg<sup>br/br</sup> CTL was ~40%, whereas by Tg<sup>gld/gld</sup> CTL it was <10%, suggesting that the amount of HA peptide presented by Renca-HA tumor cells was low (Fig. 5A). Lysis of Renca-HA cells by Tg<sup>gld/gld</sup> CTL following loading with 10<sup>−6</sup> mol/L HA peptide was increased to 90%. This lysis was blocked by Pfp inhibitor concanamycin A (Fig. 5B), suggesting that Renca-HA cells were not intrinsically resistant to Pfp-mediated lysis. In addition, Fasl-mediated lysis of Renca-HA targets, treated overnight with IFNγ and TNFα, by Tg<sup>br/br</sup> CTL was in the same range as untreated targets, suggesting that presence of IFNγ secreted by CTL in the 18-h CTL assay (Fig. 3D) was sufficient to sensitize targets to Fasl (Fig. 5B).

Lytic activity of Tg or Tg<sup>br/br</sup> CTL was also evaluated against Fas-associated death domain-like interleukin-1–converting enzyme inhibitory protein–overexpressing Renca (Renca-FLIP) cells resistant to death receptor–mediated apoptosis (18). Tg CTL lysed both control Renca-FLAG and Renca-FLIP cells loaded with 10<sup>−6</sup> mol/L HA peptide, consistent with a dominant role for Pfp-mediated lysis (Supplementary Fig. S6). By contrast, Tg<sup>br/br</sup> CTL efficiently lysed Renca-FLAG control cells loaded with either 10<sup>−6</sup> or 10<sup>−10</sup> mol/L peptide, but not Renca-FLIP cells, consistent with death ligand–mediated apoptosis being the dominant cytopathic pathway. Furthermore, cytolytic activity of Tg CTL against Renca cells loaded with 10<sup>−10</sup> mol/L HA peptide or against Renca-HA was blocked by Fasl-neutralizing mAb, but not by TNF-related apoptosis-inducing ligand (TRAIL)–neutralizing mAb (Supplementary Fig. S4B and S5). Thus, Tg<sup>/−/−</sup>–mediated lysis of Renca-HA or Renca targets loaded with low concentrations of HA peptide preferentially involved Fasl but not TRAIL or Pfp.

Sensitization of various murine and human tumor cells to Fasl-mediated lysis. To determine whether the apparent association between tumor peptide concentration and effector pathway usage was a general phenomenon, murine mammary carcinoma 4T1, colon carcinoma C-26, and B-cell lymphoma A-20 cells were loaded with different amounts of exogenous HA peptide. Addition of 10<sup>−10</sup> mol/L HA sensitized them all to lysis by Tg<sup>br/br</sup> CTL, with minimal cytolysis by Tg<sup>gld/gld</sup> CTL (Fig. 5C). At higher peptide concentrations (10<sup>−6</sup> mol/L) preferential effector pathway...
usage was lost (Fig. 5C). Following FasL-blocking mAb treatment, lysis by Tg<sup>Pfp<sup>−/−</sup></sup> CTL was reduced in 4T1 targets from 52% to 4.6% and in A20 from 75.9% to 11%.

Interestingly, treatment of human renal cell adenocarcinoma lines ACHN, TK-10, and UO-31 with recombinant FasL triggered apoptosis. This tumor cell apoptosis could be amplified by pretreatment with IFN-γ and TNF-α, and was blocked by inhibitor zVAD-FMK (Fig. 5D). Thus, FasL pathway of T-cell cytotoxicity plays a major role in cytolysis of tumor cells exposed to inflammatory cytokines such as IFN-γ and TNF-α, and becomes particularly dominant under conditions of low tumor peptide presentation.

Antigen presented by tumor targets influences the nature of CTL response and effector cell degranulation. Specific antibodies reactive to cell surface HA allowed us to sort the Renca-HA transfectants that expressed low, intermediate, and high surface levels of HA (Fig. 6A), yet similar levels of MHC class I (data not shown). Renca-HA<sup>low</sup> tumor cell lysis by Tg CTL in 18-h CTL assay was mediated predominantly by FasL pathway as it was blocked by FasL-neutralizing mAb but not by Pfp inhibitor concanamycin A (Fig. 6B). In contrast, Renca-HA<sup>high</sup> cell lysis by Tg CTL was blocked only partially by FasL-neutralizing Ab but more efficiently by concanamycin A. Lysis of unsorted Renca-HA cells and Renca-HA<sup>intermediate</sup> cells by Tg CTL showed a contribution from both effector pathways (Fig. 6C).

To understand the reason for the insensitivity of Renca-HA<sup>low</sup> targets to Pfp-mediated lysis, we investigated effector cell degranulation by measuring CD107a, a lysosomal-associated membrane glycoprotein (19). Interestingly, a significant up-regulation of


Figure 5. FasL and Pfp effector pathways play nonredundant roles. A, percent-specific lysis of Renca-HA or Renca cells, pretreated with TNF-α and IFN-γ (500 U/mL each, overnight), pulsed with various concentrations of HA peptide, and then incubated for 18 h with activated Tg, Tg<sup>Pfp<sup>−/−</sup></sup>, and Tg<sup>gld/gld</sup> CTL at effector/target ratio 25:1. *, P < 0.001 (two-sided unpaired t test) with respect to other CTL. B, percent-specific lysis of Renca-HA cells pulsed with 10<sup>−6</sup> mol/L HA peptide by Tg<sup>gld/gld</sup> CTL pretreated 2 h with medium or with concanamycin A (top). Percent-specific lysis of Renca-HA cells pretreated with medium or with TNF-α and IFN-γ (500 U/mL each, overnight) by Tg<sup>Pfp<sup>−/−</sup></sup> CTL in the presence of FasL-neutralizing mAb or its isotype control (bottom). *, P < 0.001 (two-sided unpaired t test) with respect to medium or isotype control. C, percent-specific lysis of 4T1, C-26, and A-20 tumor cells pulsed with 10<sup>−15</sup> mol/L (lo) or 10<sup>−6</sup> mol/L (hi) HA peptide at effector/target ratio 25:1. *, P < 0.001 (two-sided unpaired t test) with respect to the respective lysis values by Tg or Tg<sup>Pfp<sup>−/−</sup></sup> CTL. D, percent decrease in cell number of medium- or human TNF-α-, and IFN-γ-pretreated (100 U/mL, overnight) human renal cancer lines ACHN, TK-10, and UO-31 incubated with pan-caspase inhibitor zVAD-FMK or control analogue zFA-FMK (20 μmol/L) 1 h before the treatment with FasL (100 ng/mL). *, P < 0.001 (two-sided unpaired t test) with respect to the control. Columns and points, means (n = 3) from a representative of six (A), two (B and C), and three (D) independent experiments; bars, SD.
CD107a expression was observed in HA-specific clonotypic TCR V\(\text{h}8.1^+\) Tg CTL following incubation for 6 h with Renca-HA\text{high} targets compared with Renca-HA targets presenting lower or no HA (Fig. 6D). Thus, Renca-HA\text{high} tumor cell targets triggered degranulation in activated Tg CTL. In contrast, Renca-HA\text{low} targets failed to trigger degranulation, explaining why they are poor targets for Pfp-mediated cytolysis.

**Discussion**

We addressed the contribution of the two direct mechanisms of CD8\(^+\) T-cell cytotoxicity in the control of s.c. tumor and pulmonary metastases. In the murine model of Renca-HA cancer, where limiting levels of HA peptide are presented by tumor cells, the FasL pathway plays a dominant role in the antitumor response. Our findings provide the first clear evidence of the nonredundant antitumor roles of the Pfp and FasL effector mechanisms in vivo.

Interestingly, following the adoptive transfer of Tg CTL identical to those used in our current study, the Pfp pathway was found to be 30-fold more efficient than FasL in inducing HA-reactive autoimmune diabetes in mice with HA-expressing pancreatic \(\beta\) cells (15). Our study suggests that Renca-HA tumors are probably much more sensitive to the cytotoxic effects of FasL than pancreatic \(\beta\) cells. Endogenous cytokines may up-regulate Fas expression on tumor cells.
cells in vitro (20, 21) and further sensitize them to FasL-mediated lysis. Thus, the changes in the tumor microenvironment produced by an influx of activated CTL, coupled to the intrinsic biological characteristics of the tumor cells, seem to be crucially important in determining whether this tumor progresses or regresses. Consequently, our study explains why Pfp can be dispensable for antitumor activities of adoptively transferred CTL (10–12, 22), in certain mouse tumor models.

Previous in vitro studies have shown that self-peptides differing by one amino acid (23, 24), chemically modified peptides (25), or low concentrations of peptides (26) only act as partial agonists for cytotoxic T cells as they lead to a reduced TCR signaling. This weaker intracellular signaling triggers FasL-mediated cytotoxicity but not Pfp-mediated cytolysis (27). Our study using TCR-transgenic CD8+ T cells is in agreement with these findings. More importantly, our adoptive CTL transfer studies suggest that lower levels of tumor peptide determine the characteristics of the CTL effector response and the subsequent therapeutic outcome. We would speculate that many tumors will not trigger engagement of the Pfp-mediated cytotoxic pathway in vitro, even if the T cells have all the relevant lytic machinery available to them.

We found no role of FasL-mediated cytotoxicity in the “bystander” lysis of Ag-deficient Renca targets by TgPfp+/−/−CTL (data not shown), suggesting that the eradication of tumor by Pfp−/−/−CTL seemingly requires a direct tumor/effector contact. Antitumor effects involving tumor stroma are unlikely, as evident from the efficient FasL-dependent rejection of Renca-HA tumor in Fas+/−/− mice where the stroma would be resistant to Fasl. However, the role of stromal cells in tumor rejection may be dependent on the tumor site or type. Cytokines such as IFN-γ or TNFα have been reported to have effects on tumor stroma in promoting tumor rejection in some models (28, 29). We found also no role for TRAIL in Renca-HA lysis by HA-reactive CTL, although Renca cells are sensitive to TRAIL (18, 30).

The FasL-mediated tumor eradication may also serve as an immunoselective pressure to select Fas-deficient/low and/or Ag-negative tumor cells. This immunoselection has been reported in the context of CD4+ T cell-mediated response against Renca-HA tumor (31) and in other tumor models (32). We have also observed that the s.c. Renca-HA tumors growing out in WT mice become HA and/or Fas negative.4 The ultimate success of the antitumor T-cell response may thus rely not only on the efficacies of Pfp and FasL pathways to control the continuum of high Ag to low antigenic targets but also on various other mechanisms including activation of innate immune cells that infiltrate the tumor. Recently, the antitumor CTL response was shown to activate local natural killer cells to eliminate Ag-loss tumor variants (33). Tumor destruction in vivo, by mechanisms other than direct CTL-mediated cytotoxicity, is worthy of further investigation.

This study suggests that biological characteristics of the tumors, including their FasL sensitivity and the quantity of antigen presented in association with MHC class I, will play a major role in determining the efficacy of immunotherapy. Interestingly, some human renal carcinoma lines are susceptible to the apoptotic effects of Fasl in vitro. In addition, pharmacologic agents such as the proteasome inhibitor bortezomib can sensitize tumor cells to apoptosis (30, 34). It is thus tempting to speculate that certain human renal carcinomas that are can be made sensitive to FasL-mediated apoptosis would be more responsive to adoptive T-cell therapy. Promotion of FasL-mediated T-cell cytotoxicity in combination with other therapeutic strategies that sensitize tumor cells to apoptosis may thus offer significant future potential for effective cancer immunotherapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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