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Tumor-Specific CTL Kill Murine Renal Cancer Cells Using Both Perforin and Fas Ligand-Mediated Lysis In Vitro, But Cause Tumor Regression In Vivo in the Absence of Perforin[1]

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Kidney cancer is a devastating disease; however, biological therapies have achieved some limited success. The murine renal cancer Renca has been used as a model for developing new preclinical approaches to the treatment of renal cell carcinoma. Successful cytokine-based approaches require $CD8^+$ T cells, but the exact mechanisms by which T cells mediate therapeutic benefit have not been completely identified. After successful biological therapy of Renca in BALB/c mice, we generated CTLs in vitro using mixed lymphocyte tumor cultures. These CTL mediated tumor-specific H-$2K^d$-restricted lysis and production of IFN-γ, TNF-α, and Fas ligand (FasL) in response to Renca. CTL used both granule- and FasL-mediated mechanisms to lyse Renca, although granule-mediated killing was the predominant lytic mechanism in vitro. The cytokines IFN-γ and TNF-α increased the sensitivity of Renca cells to CTL lysis by both granule- and FasL-mediated death pathways. Adoptive transfer of these anti-Renca CTL into tumor-bearing mice cured most mice of established experimental pulmonary metastases, and successfully treated mice were immune to tumor rechallenge. Interestingly, we were able to establish Renca-specific CTL from mice gene targeted for perforin ($pfp^{-/-}$) mice. Although these $pfp^{-/-}$ CTL showed reduced cytotoxic activity against Renca, their IFN-γ production in the presence of Renca targets was equivalent to that of wild-type CTL, and adoptive transfer of $pfp^{-/-}$ CTL was as efficient as wild-type CTL in causing regression of established Renca pulmonary metastases. Therefore, although granule-mediated killing is of paramount importance for CTL-mediated lysis in vitro, some major in vivo effector mechanisms clearly are independent of perforin. ***The Journal of Immunology*****, 2002, 168: 3484–3492.**

Some human renal cell carcinomas (RCC)[4] as well as the Renca mouse RCC respond to cytokine-based immunotherapies. In particular, regimens containing IL-2 have shown efficacy in both man and mouse (1). In the mouse, regimens containing IL-2 plus the cytokine-inducing flavone acetic acid or IL-12 plus IL-2 (IL-12 pulse IL-2) show impressive therapeutic benefits against established metastatic Renca (2, 3). For these studies, $CD8^+$ T cells have been consistently implicated as critical components for tumor regression in vivo. However, the nature of the requisite effector functions of T cells critical for therapeutic benefits in vivo have not been identified, although the generation of IFN-γ was also found to be essential for the success of immunotherapy (4).

Perforin-mediated cytotoxicity and Fas/Fas ligand (FasL)-interaction are major mechanisms for $CD8^+$ T cell-mediated effector function (5, 6). After the TCR engages specific antigenic peptides presented by the MHC of target cells, perforin is released and causes damage to target cell membranes. Various granzymes, and possibly other granule constituents cosecreted with perforin, enter the target cells and induce apoptosis (7). The expression of a variety of cytokines, including FasL, is also enhanced in the T cells after Ag-specific activation. FasL cross-links Fas on the target cell, and in many cells this interaction triggers apoptosis and cell death (6). It has been reported that other members of the TNF family can also be expressed by antitumor effector cells. For example, TNF-related apoptosis-inducing ligand (TRAIL) can interact with its receptors on tumor cells and cause tumor cell death (8). However, the extent to which these cytotoxic mechanisms are required for CTL-mediated tumor destruction in vivo remains controversial, and the relative importance of granule vs FasL-mediated lytic activities are not well understood.

Studies on tumor surveillance in perforin-deficient mice have demonstrated that perforin-dependent cytotoxicity was crucial for resistance against injected tumor cell lines, viral and chemical carcinogenesis as well as spontaneous leukemogenesis (9–11), while FasL was reported to play only a minor role in this setting. Similarly, studies in tumor-bearing, perforin-deficient mice have also shown a major contribution of perforin to regression of established tumors (12, 13). However, many of the tumors used in these previous studies were intrinsically resistant to Fas-mediated killing

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[4] Abbreviations used in this paper: RCC, renal cell carcinoma; TRAIL, TNF-related apoptosis-inducing ligand; FasL, Fas ligand; P/I, PMA plus ionomycin; [^{111}In]Ox, ^{111}In-labeled oxine; TDLN, tumor-draining lymph node; CMA, Concanamycin A; WT, wild type; BLT, *N*-a-benzyloxycarbonyl-L-lysinethiobenzoyl ester.

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(9), and some of these experimental models used xenotransplantation of human tumors into mice (12, 13). In such experimental systems, the species-specific activities of some cytokines and the strength of xenogeneic immune responses may mask relevant biological roles for cytokines and FasL-mediated effects. Relatively strong antigenic stimuli such as xenoantigens may induce antitumor responses qualitatively and quantitatively different from spontaneously arising tumors. More recent studies have shown that Fas-associated death domain-like IL-1β-converting enzyme inhibitory protein-transfected tumors grew more rapidly than control transfectants in wild-type (WT) mice, whereas growth rates in SCID mice were equivalent (14, 15). Control and FLIP transfectants were lysed equally well in vitro by CTL using granule-mediated lytic pathways, whereas the FLIP transfectants were much more resistant to FasL-mediated lysis. These studies suggested that Fas-dependent apoptosis constituted a more prominent mechanism for tumor clearance in vivo than had previously been anticipated. Furthermore, Lee et al. (16) demonstrated that Renca cells overexpressing Fas grew at much slower rates than control transfectants in vivo, further indicating that FasL-mediated lysis could be an important effector mechanism for tumor surveillance by T cells against some tumors.

A number of experimental murine tumors are resistant to FasL-mediated lysis in vitro, making it unlikely that this lytic mechanism constitutes an important antitumor effector pathway for destruction of these tumors in vivo (9). However, some studies have reported that Fas expression can be enhanced on certain murine tumors by in vivo tumor passage (17). In this situation, it is possible that in the in vivo milieu FasL-mediated antitumor effects may be more important than would be predicted from in vitro assays. In this study, we established CTL specific for in vitro reactivity to Renca from mice successfully treated with flavone acetic acid plus IL-2 or IL-12 pulse IL-2. The molecular mechanisms used by these CTL to lyse Renca tumor cells in vitro and cause regression of Renca metastases in vivo were then analyzed.

Materials and Methods

Mice

Specific pathogen-free BALB/c mice were obtained from the Animal Production Area, National Cancer Institute (Frederick, MD). Perforin-deficient (BALB/c pfp$^{-/-}$) mice were kindly provided by Dr. M. Smyth (Peter MacCallum Cancer Institute, Melbourne, Australia) and bred in our specific pathogen-free facility. Animal care was provided in accordance with the procedures outlined in *A Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 86-23; 1985).

Cell lines

The Renca tumor cell line is of BALB/c origin. The following cell lines were used as target cells for CTL assays: stretoxotocin-induced renal cell carcinoma-1, a recently derived, streptozotocin-derived RCC of BALB/c origin (18); A20 B cell lymphoma; the C26, a colon cancer of BALB/c origin; the MethA fibrosarcoma of BALB/c origin; L10A leukemia of BALB/c origin; the Harvey BALB/c fibroblast cell line transformed with the Harvey Ras oncogene (kindly provided by Dr. R. Hornung, Science Applications International Corporation, Frederick, MD); P815 mastocytoma (DBA/2); the B16 melanoma (C57BL/6); and the L929 fibrosarcoma (C3H/An). All the lines were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1× nonessential amino acids, 1 mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 μg/ml), 10 mM HEPES, and 5×10^{-5} M 2-ME, pH 7.4 (complete medium). Con A blasts (19) and fibroblasts were prepared from spleen cells as previously described.

Reagents

Recombinant human IL-2, recombinant murine IL-12, and murine rIFN-γ were generously provided by Chiron (Emeryville, CA), Genetics Institute (Cambridge, MA), and Genentech (South San Francisco, CA), respectively. Murine rTNF-α was purchased from BD PharMingen (San Diego, CA). Human rIFN-α A/D was kindly provided by Dr. M. Brunda (Hoffmann-LaRoche, Nutley, NJ). Human rIL-15 and murine rIL-18 were obtained from PeproTech (Rocky Hill, NJ). The following Abs were purchased from BD PharMingen: mAbs against mouse H-2K^d (SF1-1.1), H-2D^d (34-5-8AS), H-2L^d (28-14-8), CD4 (GK1.5), CD8 (53-6.7), DX-5, TNF-α (MP6-XT22), IFN-γ (XMG1.2), and LFA-1 (M17/4). An anti-mouse ICAM-1 (KAT-1) was purchased from R&D Systems (Minneapolis, MN). Anti-mouse FasL (MFL-1) and anti-mouse TRAIL (N2B2) mAb were kindly provided by Dr. H. Yagita (Juntendo University, Tokyo, Japan; Ref. 20). PMA and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO).

Establishment and maintenance of CTL line

Renca tumor cells (10^5) were injected under the kidney capsule of BALB/c mice. Mice were then treated on days 7–9 after tumor inoculation with FAA and IL-2, as previously described (2). In some experiments, BALB/c tumor-bearing mice treated with a combination of IL-2 and IL-12 (3) were used as a source of CTL. Mice surviving >30 days after therapy were rechallenged i.v. with 10^5 Renca cells. Splenocytes isolated 1 wk later were stimulated in vitro with IFN-γ-treated (200 U/ml) and irradiated (100 Gy) Renca cells at a splenocyte:tumor cell ratio of 20:1 in the presence of IL-2 (10 U/ml). CTL were established and maintained in complete media supplemented with IL-2 (10 U/ml). Viable cells (5×10^5) were maintained as bulk CTL lines by weekly restimulation with irradiated Renca cells (5×10^4) and irradiated (20 Gy) splenic feeder cells of BALB/c mice ($4–5 \times 10^6$) in 24-well culture plates for the following experiments. CTL were also established from BALB/c pfp$^{-/-}$ mice in an identical manner.

Flow cytometric analysis

Flow cytometry analysis was performed on a FACScan (BD Biosciences, Mountain View, CA) using CellQuest software. For the detection of FasL or TRAIL on CTL, CTL (2×10^6 cells/ml) were cultured at 37°C for 6, 12, or 20 h with the following agents in media supplemented with a matrix metalloproteinase inhibitor (10 μg/ml, KB8301; BD PharMingen): IFN-α (200 U/ml), IFN-γ (500 U/ml), TNF-α (100 ng/ml), IL-2 (500 U/ml), IL-12 (20 ng/ml), IL-15 (150 ng/ml), IL-18 (500 ng/ml), plate-coated anti-CD3 mAb (10 μg/ml), or PMA (20 ng/ml) plus ionomycin (1 μg/ml) (P/I). Alternatively, CTL were cultured with 10^5 Renca cells or Harvey BALB fibroblasts. These CTL (1×10^6) were incubated with l μg of biotinylated anti-FasL, anti-TRAIL mAb, or isotype control (hamster IgG for FasL and rat IgG2a for TRAIL; BD PharMingen) for 30 min at 4°C followed by PE-labeled avidin. After washing with PBS, the cells were analyzed on a FACScan.

Cell surface markers of Renca were analyzed after overnight treatment with IFN-γ (500 U/ml) and/or TNF-α (400 U/ml) or media alone by flow cytometry using FITC-labeled mAbs reactive to H-2K^d (SF1-1.1), ICAM-1 (3E2), LFA-1(M17/4), CD48(HM48-1), CD80 (16-10A1), CD86 (GL-1), ICAM-2 (3C4), LFA-1 (M17/4), CD48 (HM48-1), CD80 (16-10A1), CD86 (GL-1), and PE-labeled mAb reactive with Fas (Jo2), or isotype-matched mAbs. All mAbs were purchased from BD PharMingen.

Cytotoxicity assays

Renca cells that had been incubated overnight in the presence or absence of IFN-γ (500 U/ml) and TNF-α (400 U/ml) were labeled with ^{111}In-labeled oxine ([^{111}In]Ox; Medi-Physics, Silver Spring, MD) as previously described (19). Other target cells were labeled with [^{111}In]Ox in the same manner. Briefly, 1×10^6 target cells were incubated with 10 μCi of [^{111}In]Ox for 30 min at room temperature. Cells were then washed twice in complete medium and labeled cells (1×10^4) were then incubated with effector CTL at various E:T ratios for 6–8 or 18 h at 37°C in a final volume of 200 μl. Short-term cytotoxicity assays of 6–8 h were used in preference to standard 4-h assays which, although adequate for granule-mediated killing, tend to underestimate death receptor-mediated lysis. For inhibition experiments, various mAbs were added to the culture at a final concentration of 10 μg/ml. For the inhibition of FasL- or TRAIL-dependent cytotoxicity, CTL activity was tested in the presence of a neutralizing anti-FasL mAb (MFL-1) or anti-TRAIL mAb (N2B2) at 10 μg/ml, respectively. For inhibition of perforin-dependent cytotoxicity, CTL were pretreated with Concanamycin A (CMA; Sigma-Aldrich) for 2 h at 100 nM, and then cytotoxic assays were performed in the continuous presence of CMA. After incubation, supernatants were harvested and counted on a gamma counter. Specific killing (percentage of cytotoxicity) was calculated as: [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100. All groups were run in triplicate, and all experiments were performed three or more times with similar findings.

Cytokine ELISA

CTL (4×10^4 cells) were incubated with 10^4 stimulator cells (E:T = 4:1) or media alone in 96-well plates at 37°C for 16 h. For the blocking experiments, mAb was added to the culture at a final concentration of 10 μg/ml. Cell-free supernatant was harvested and subjected to IFN-γ, TNF-α, or IL-4 ELISA (R&D Systems; sensitivity limits: 2 pg/ml).

N-*a-benzyloxycarbonyl-L-lysine thiobenzoyl ester (BLT) esterase release assay*

CTL (1×10^5) were incubated with Renca or IFN-γ/TNF-α-treated Renca (1×10^4) for 4 h in 96-well tissue culture plates with or without anti-LFA-1 mAb. The BLT activity of the supernatants was measured as previously described (21).

Adoptive transfer of CTL in vivo

Treatment of pulmonary metastasis model was initiated 3 days after the i.v. injection of 1.5×10^5 Renca cells into syngeneic BALB/c WT mice or BALB/c pfp$^{-/-}$ mice. CTL derived from BALB/c WT or BALB/c pfp$^{-/-}$ mice were transferred i.v on day 3. In initial experiments, CTL or Con A blast T cells as a control were transferred i.v. at 10^7 cells per day on days 3, 4, and 5 together with IL-2 (10,000 U/day). In later experiments, CTL were simply transferred on day 3 in the absence of IL-2 and survival was monitored, or cohorts of mice were euthanized on day 17, and numbers of lung metastasis were counted.

Statistical analysis

The significance of difference in number of metastasis between experimental groups was determined by the Mann-Whitney *U* test. Two-sided *p* values of <0.05 are considered significant. Survival data was analyzed by the log rank test, and *p* values of <0.01 were considered significant.

Results

Generation of anti-Renca CTL

Renca cells were injected under the renal capsule of BALB/c mice, followed by immunotherapy with FAA plus IL-2. The majority (>80%) of mice undergoing this therapy survived and were rendered tumor-free, whereas all untreated mice died between 20 and 27 days from extensive tumor development. Splenocytes from surviving mice were isolated 1 wk after i.v. rechallenge with tumor, and CTL were generated by mixed lymphocyte tumor culture in vitro. Seven days after the in vitro culture was started, cells were >95% CD3$^+$, CD8$^+$, CD4$^-$, TCR$\alpha\beta^+$, and DX5$^-$ T cells by FACS analysis (data not shown). These enriched CD8 T cells were maintained as a CTL line by weekly restimulation with Renca cells and splenic feeder cells, and used in subsequent experiments.

Established CTL were examined for cytotoxicity and cytokine production in vitro in response to various cell lines (Table I). CTL showed specific lysis of Renca but not other syngeneic (BALB/c) or allogenic cell lines. In addition, CTL showed no lytic activity against autologous fibroblasts or Con A splenic T cell blasts. IFN-γ and TNF-α were also produced only in response to Renca. In contrast, CTL showed no production of IL-4 or IL-12 in response to any stimulator cells (data not shown). These specific responses against Renca were inhibited with anti-CD8 or anti-H2K^d blocking mAb, but not by either anti-CD4 or anti-H-2D^d mAb, clearly demonstrating the Renca specificity and H-2K^d restriction of these CTL.

Interestingly, CTL showed cytotoxic activity against Renca only in relatively long-term (18 h), but not in short-term cytotoxicity assays (8 h). However, if Renca cells were pretreated for 18 h with IFN-γ and TNF-α before the cytotoxicity assay, these cells became very susceptible to CTL lysis even in short-term assays (Fig. 1*A*). This indicates that cytokines routinely produced during the coincubation of CTL and tumor cells could play an important role in sensitizing Renca to CTL lysis. This hypothesis was confirmed by inhibition of CTL lysis by neutralizing mAbs to IFN-γ and/or TNF-α (Fig. 1*B*). CTL-mediated lysis of Renca cells was partially inhibited with anti-TNF mAb in the long term assays, while the lysis was almost completely inhibited with either anti-IFN-γ mAb or the combination of anti-IFN-γ and anti-TNF-α mAb. In contrast, these neutralizing mAb did not inhibit CTL lysis against Renca cells that were pretreated with IFN-γ and TNF-α before the assay. These results suggest that cytokines produced by the CTL on contact with Renca cells can ultimately sensitize Renca cells to lysis by these CTL. Most of the CTL lysis of Renca cells in vitro was blocked by the perforin inhibitor CMA (22) in both short- (66.5% inhibition) and long-term assays (78.2%), suggesting a major effector role for granule-mediated lysis in vitro (Fig. 1*A*). However, some inhibition of lysis was also observed when Abs to FasL were present. Furthermore, almost all lysis was inhibited with the combination of CMA and anti-FasL-neutralizing mAb. We also observed that Renca was resistant to any cytotoxic effects of TNF-α in vitro even at doses of up to 10,000 U/ml (data not shown). In addition, anti-TRAIL mAb did not inhibit CTL lysis of Renca, even though Renca is known to be susceptible to TRAIL-mediated killing (19). These findings suggest that the vast majority of CTL-mediated lysis of Renca cells in vitro could be accounted

Table I. *Cytotoxicity and cytokine production by CTL on coincubation with various cell lines*[a]

	Histology	H-2^{db}	mAb	Lysis (%)	IFN-γ (pg/ml)	TNF-α (pg/ml)
Renca	Renal cell cancer	+	–	54	>8,000	488
			Anti-CD4	57	>8,000	441
			Anti-CD8	12	0	0
			Anti-H2-K^d	16	931	97
			Anti-H2-D^d	45	>8,000	527
STRCC-1	Renal cell cancer	+	–		0	
P815	Mastocytoma	+	–	4	0	0
C26	Colon cancer	+	–	0	0	0
MethA	Fibrosarcoma	+	–	1	3	
L10A	Lymphoma	+	–	0	13	0
A20	Leukemia	+	–	2	15	0
Harvey BALB	Fibroblast	–	–	0	0	0
Fibroblast		+	–	5	1	0
ConA splenic blast		+	–	0		
B16	Melanoma	–	–	2	0	0
L929	Fibrosarcoma	–	–	3	4	

[a] Cytotoxicity against various lines was measured in a 16 h ^{111}In-release assay at an E:T ratio of 20. CTL and stimulator cells were cultured for 16 h (E/S = 4), and IFN-γ and TNF-α release of supernatant was measured by ELISA. For the inhibition experiments, blocking mAb was added to the culture at the concentration of 10 μg/ml. The representative data of three individual experiments are shown.

[b] Expression of H-K^d molecule on cell surface was tested by flow cytometer analysis.

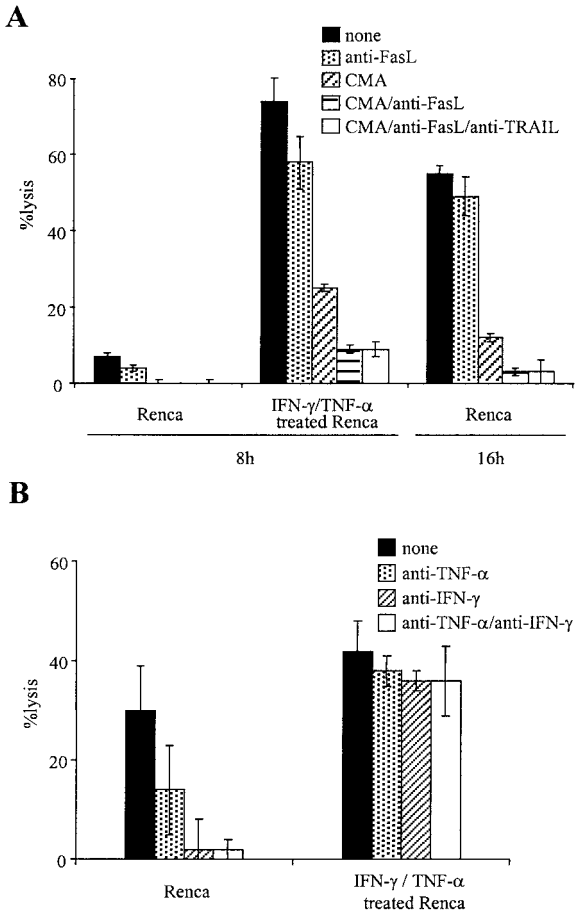

FIGURE 1. Effect of inhibitors on T cell lysis of Renca. Cytotoxic activity of CTL was tested against Renca or Renca pretreated with IFN-γ (500 U/ml) and TNF-α (400 U/ml) by [^{111}In]Ox release assay. *A*, The 8- or 18-h cytotoxicity assays (E:T = 20:1) were performed in the presence or absence of CMA (100 nM), anti-FasL, anti-TRAIL mAb (10 μg/ml), or combinations of these agents. *B*, An 18-h assay (E:T = 2:1) was performed in the presence or absence of anti-IFN-γ, anti-TNF-α mAb (10 μg/ml), or both of these mAbs. Data represent mean ± SD of triplicate samples. Similar results were obtained in three independent experiments.

for by either granule- or FasL-mediated mechanisms. Because cytokine pretreatment sensitized Renca cells to the lytic effects of the CTL, further studies were performed to determine the molecular basis of this increased sensitivity to cytolysis.

Importance of the interaction between ICAM-1 and LFA-1 for perforin-mediated killing

IFN-γ and TNF-α enhanced susceptibility of Renca to CTL lysis as shown in Fig. 1. Therefore, the effects of IFN-γ and TNF-α on Renca target cells were examined in more detail in Fig. 2. Renca cells constitutively express very low levels of Fas; however, treatment with either IFN-γ or TNF-α up-regulated Fas expression, and the combination of these cytokines synergistically enhanced surface Fas expression on Renca. The enhanced Fas induction by this cytokine combination sensitized of Renca to FasL mediated killing by CTL, as previously reported (23). In addition, ICAM-1 is only expressed at low levels on Renca, but IFN-γ pretreatment induced high levels of surface expression. TNF-α induced only a slight increase in ICAM-1 expression, and the combination of IFN-γ with TNF-α enhanced ICAM-1 induction more than either cytokine alone. Renca cells express high endogenous levels of MHC class I, and IFN-γ and the combination of IFN-γ plus TNF-α further enhanced expression of the H-2K^d molecule on Renca cells. CD11a (LFA-1), CD48 (CD2 ligand), CD80 (B7.1) CD86 (B7.2), and CD102 (ICAM-2) were neither constitutively expressed nor induced after the cytokine treatment (data not shown). In short-term cytotoxicity assays, most of the CTL killing of Renca cells that were pretreated with these cytokines was inhibited with anti-ICAM-1 mAb and anti-LFA-1 mAb (Fig. 3*A*), suggesting that in-

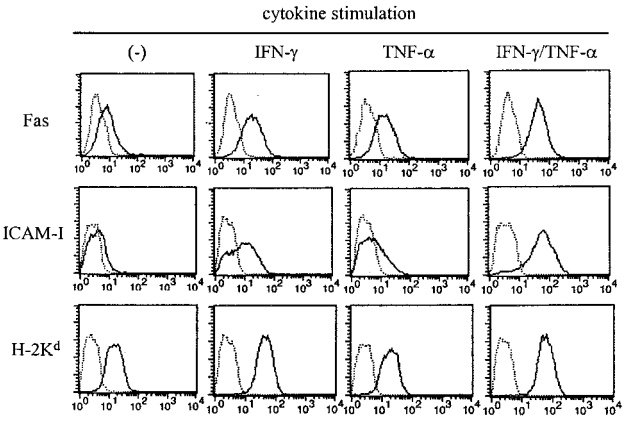

FIGURE 2. Renca was cultured overnight in the presence or absence of IFN-γ (500 U/ml), TNF-α (400 U/ml), and the combination of these two cytokines. These cells were then stained with PE-labeled anti-Fas, or FITC-labeled anti-H-2K^d or ICAM-1 mAb (solid lines). Dotted lines show background staining with isotype-matched control mAbs. Solid lines show staining with test Abs.

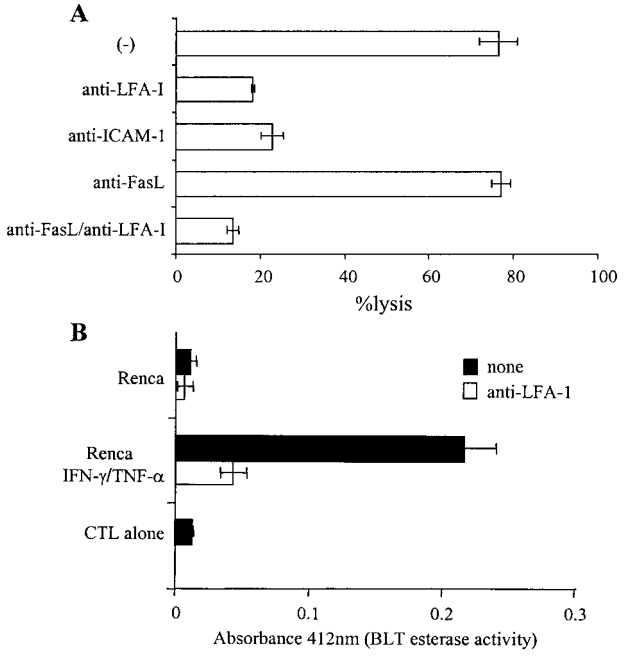

FIGURE 3. Inhibition of CTL activities to Renca by blocking of LFA-1/ICAM-1 interactions. *A*, Renca pretreated for 18 h with IFN-γ (500 U/ml) and TNF-α (400 U/ml) were used as targets in a 6 h [^{111}In]Ox release assay (E:T = 20:1) in the presence or absence of anti-LFA-1, anti-ICAM-1, and anti-FasL mAbs (10 μg/ml), or the combination of anti-LFA-1 and anti-FasL mAbs. *B*, Release of BLT esterase by CTL. CTL (1×10^5) were incubated with Renca or IFN-γ/TNF-α-treated Renca (1×10^4) for 4 h in 96-well tissue culture plates with or without anti-LFA-1 mAb (10 μg/ml). Next, the BLT activity in the supernatants was measured. Data represent mean ± SD of triplicate samples. Similar results were obtained in three independent experiments.

teraction of ICAM-1 on Renca and LFA-1 on CTL may be a crucial requirement for granule-mediated killing of Renca cells. Lysis in this short-term assay was predominantly granule-mediated, since anti-FasL had little effect. Degranulation of CTL was also estimated by the release of BLT esterase activity (Fig. 3*B*). BLT esterase is stored in the granules together with perforin/granzymes, and its secretion correlates with the exocytosis of lytic granules. Supernatants from the culture of CTL and Renca cells that had been pretreated with IFN-γ and TNF-α showed higher levels of BLT esterase activity than those from CTL cultured with untreated Renca. A large part (80%) of this released BLT esterase activity was inhibited when anti-LFA-1 mAb was present during the CTL and Renca incubation (Fig. 3*B*). Anti-ICAM-1 could also block degranulation, but was usually less efficient than anti-LFA-1 (data not shown). Binding assays using isotope-labeled effector cells also showed that specific binding between cytokine-treated Renca and CTL was 4–5 times stronger than binding between untreated Renca and CTL. This increased binding capacity was also inhibited with anti-LFA-1 mAb (data not shown). Collectively, this suggests that ICAM-1 and LFA-1 interactions enhanced the binding between CTL and Renca cells. This resulted in an amplification of signals though the TCR, such that degranulation was increased. Therefore, the cytokines IFN-γ and TNF-α sensitize Renca to CTL lysis by at least two mechanisms: the increase of Fas expression on Renca is crucial for the optimal lytic effects of FasL, and the induction of ICAM on Renca enhances the binding between CTL and Renca cells, which increases degranulation of CTL.

Tumor lysis via death receptors

Because both FasL and TRAIL have been reported to lyse Renca cells (19, 23–25), we investigated the stimuli that control FasL and TRAIL expression on anti-Renca CTL. FasL expression was upregulated on CTL after 6 h specific interaction with Renca cells, but not with the syngeneic Harvey BALB cell line (Fig. 4). CTL also expressed high levels of FasL after stimulation with P/I or with immobilized anti-CD3 mAb (data not shown). In contrast, cytokine treatment of CTL with IL-2, IL-12, and IFN-α had minimal effects on CTL expression of FasL (data not shown). TRAIL was only weakly induced on CTL after incubation with Renca tumor cells or P/I, indicating FasL and TRAIL are differentially regulated in these CTL. Interestingly, only IFN-α could significantly induce TRAIL on CTL, while other cytokines including IL-2 and IL-12 only had modest effects. The cytokines IL-15, IL-18, IFN-γ, and TNF-α had minimal effects on either FasL or TRAIL expression by CTL (data not shown). These patterns of FasL and TRAIL expression remained consistent when the same stimuli were used for 12 or 20 h (data not shown).

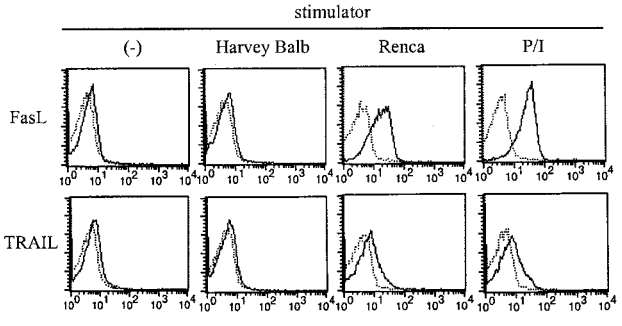

FIGURE 4. Regulation of FasL and TRAIL expression on CTL by various stimuli. CTL were cocultured with Renca, Harvey BALB fibroblasts, or the combination of PMA (20 ng/ml) and ionomycin (1 μg/ml) for 6 h. These CTL were stained with biotinylated anti-FasL or anti-TRAIL mAb and PE-avidin (solid lines). Dotted lines show background staining with biotinylated isotype control and PE-avidin; solid lines show staining with test Abs.

Adoptive transfer of CTL to tumor-bearing mice

Because Renca-specific CTL produced high levels of cytokines and were highly lytic in response to Renca cells in vitro, we next tested their antitumor efficacy after transfer to tumor-bearing BALB/c mice. CTL specific for Renca cells were tested for therapeutic efficacy against established experimental pulmonary metastases. In this experimental metastasis model, CTL were transferred i.v. on days 3–5 together with 10,000 U IL-2. Control mice were treated with an equivalent number of Con A blasts (>95% $CD3^+$ and $CD8^+$) plus IL-2, or with IL-2 alone. A portion of the mice were then sacrificed on day 17, and numbers of lung metastasis were counted (Fig. 5*A*). Mice treated with IL-2 alone showed a large number of metastasis (>200), whereas mice receiving specific CTL transfer showed zero or very few metastases (n = 0–5). In mice receiving Con A blasts, metastases (n = 125–200) were significantly lower than in mice receiving IL-2 alone, but were still numerous when compared with the CTL-treated mice. Furthermore, most mice receiving specific CTL survived >60 days, while all mice receiving Con A blasts or IL-2 were dead within 30 days of tumor injection (Fig. 5*B*). Because CTL therapy was very effective, we determined the minimal number of CTL required for adoptive therapy. One injection of 1×10^6 CTL in the absence of IL-2 provided complete therapeutic benefit, whereas 2×10^5 CTL could substantially reduce the number of metastases (by >80%),

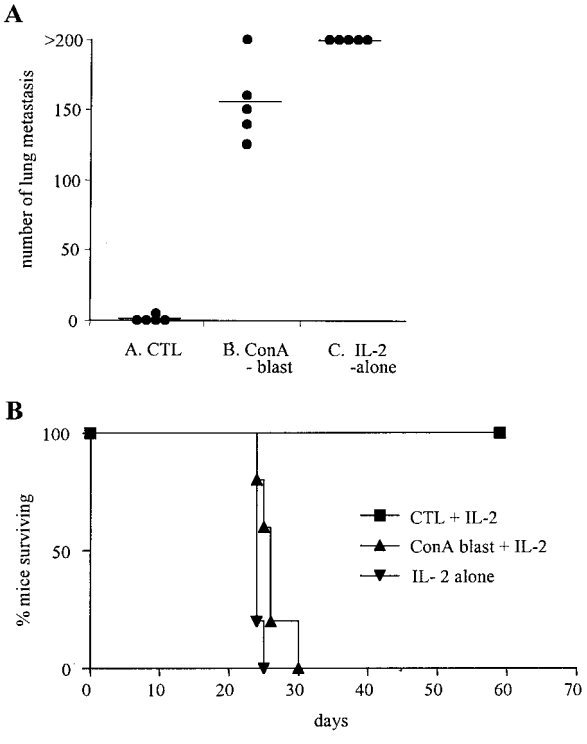

FIGURE 5. *A*, Adoptive transfer of CTL in 3-day experimental metastasis model. Renca cells (1.5×10^5) were injected i.v. to BALB/c mice on day 0. CTL or Con A blasts (10^7 cells/day) with IL-2 (10,000 U/day) or IL-2 alone were administered on days 3, 4, and 5. On day 17, half of the mice were sacrificed and lung metastasis was quantified. ●, A single mouse. A horizontal bar shows the average number of metastases in each group. A vs B, $p < 0.01$; A vs C, $p < 0.01$; B vs C, $p < 0.05$. *B*, Survival of the remainder of the mice was monitored. The data shown are representative of two independent experiments.

but did not provide complete therapeutic benefits (data not shown). Mice that survived adoptive CTL transfer were immune to rechallenge with the Renca tumor (data not shown).

Generation of CTL from pfp$^{-/-}$ mice and adoptive transfer of pfp$^{-/-}$ CTL

CTL use both granule-mediated and FasL-mediated pathways to lyse Renca cells in vitro, and the granule-mediated pathway seems to be the predominant lytic mechanism. To investigate the importance of the granule-mediated pathway in vivo, CTL were established from pfp$^{-/-}$ mice. CTL were generated by in vitro mixed lymphocyte tumor cultures with spleen cells from pfp$^{-/-}$ mice that had been successfully treated with FAA plus IL-2, in the same way that CTL were successfully isolated from WT mice. As expected, pfp$^{-/-}$ CTL showed a reduced lytic activity against Renca cells in vitro (Fig. 6*A*), but produced equivalent amounts of IFN-γ (Fig. 6*B*) and TNF-α (data not shown) upon stimulation with Renca, but not with syngeneic A20 cells. Both lytic activity and cytokine production by pfp$^{-/-}$ CTL were blocked with anti-CD8. These results demonstrate the specificity and the expected MHC restriction of the pfp$^{-/-}$ CTL. Furthermore, anti-FasL blocked all lysis by pfp$^{-/-}$ CTL, demonstrating that granule-mediated lysis was absent in these CTL (Fig. 6*C*). We have previously shown that cytokine-induced ICAM-1 expression on the Renca target cells was important for degranulation of CTL. Using pfp$^{-/-}$ CTL, we determined whether ICAM-1 expression enhanced FasL-mediated lysis. An 8 h cytotoxic assay with IFN-γ and TNF-α pretreated Renca cells was performed (Fig. 6*D*), and lytic activity of pfp$^{-/-}$ CTL was partially inhibited with anti-ICAM-1 or anti-LFA-1 mAb, and almost completely abrogated with the combination of anti-LFA-1 and anti-FasL mAbs. These findings suggest that the interaction of ICAM-1 on Renca cells and LFA-1 on CTL increases the lytic efficiency of the FasL pathway by 30–50%.

CTL derived from WT or pfp$^{-/-}$ mice were transferred 3 days after i.v. tumor injection in the absence of IL-2. We had previously determined that as few as 1–2 $\times$ 10^6 WT CTL administered i.v. produced complete tumor regression of pulmonary metastasis in the absence of IL-2. Numbers of lung metastases, which correlates well with ultimate survival (data not shown), were counted on day 17. In mice receiving either WT or pfp$^{-/-}$ CTL, zero or very few metastases were observed at two different doses of CTL (5 $\times$ 10^6 and 2 $\times$ 10^6 cells). In contrast, a large number of metastases developed in control mice treated with vehicle alone (Fig. 7).

There remained the possibility that adoptive transfer of CTL could have generated host-derived effector T or NK cells that contained perforin from the BALB/c WT recipients. To exclude this possibility, pfp$^{-/-}$ CTL were transferred to pfp$^{-/-}$ mice (Fig. 8*A*). No lung metastases were observed on day 17 in CTL-treated mice using either BALB/c or BALB/c pfp$^{-/-}$ recipients, while large numbers of metastases were observed in vehicle-treated mice. Furthermore, BALB/c or BALB/c pfp$^{-/-}$ mice receiving CTL survived >60 days after tumor challenge, whereas control mice all died within 30 days (Fig. 8*B*). These results clearly show that the granule-mediated pathway is not essential for the regression of Renca pulmonary metastases after the adoptive transfer of CTL in vivo. Interestingly, we also consistently observed that pfp$^{-/-}$ mice developed fewer lung metastases than WT mice; however, the reason for this remains unclear. Renca-specific CTL generated from WT and pfp$^{-/-}$ mice successfully treated with IL-12 pulse IL-2 therapy gave identical results for the treatment of established experimental pulmonary metastases. Overall, these results unequivocally demonstrate a clear dichotomy between tumor-specific lysis of tumor cells in vitro and rejection of tumor in vivo. The results implicate non-pfp-dependent mechanisms that may include Fas/FasL interactions as critical events in the regression of pulmonary Renca metastases.

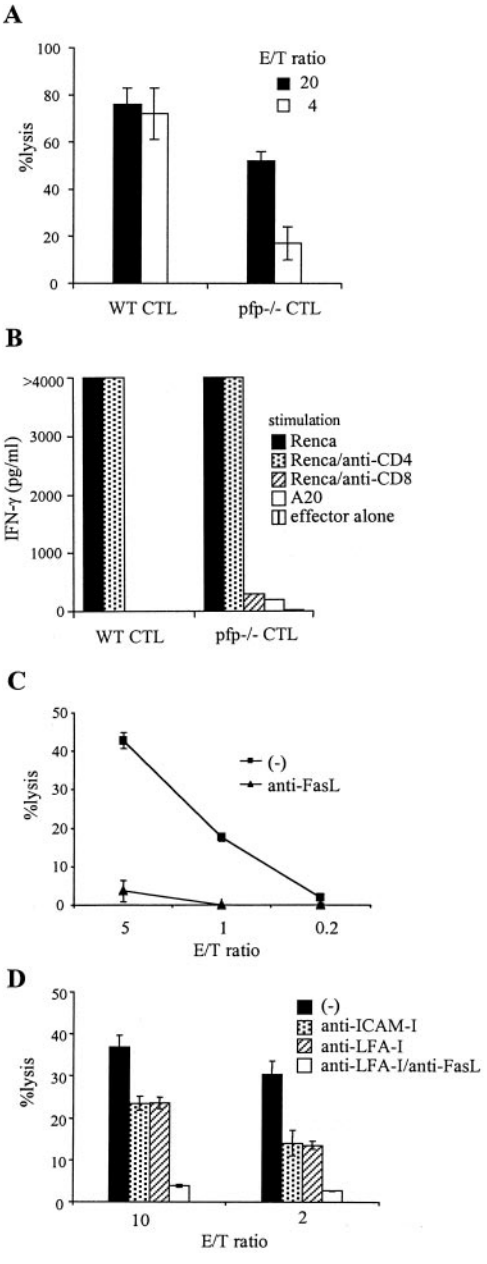

FIGURE 6. Activity of CTL generated from pfp$^{-/-}$ mouse. *A*, Cytotoxic activities of CTL generated from WT mouse and pfp$^{-/-}$ mouse against Renca were tested in an 18 h [^{111}In]Ox release assay (E:T = 20 or 4:1). *B*, Specific IFN-γ production. CTL generated from WT or pfp$^{-/-}$ mice were incubated with Renca, A20, or medium alone for 18 h at an E:T of 4:1 in the presence or absence of anti-CD4 or anti-CD8 mAb (10 μg/ML). The IFN-γ in the supernatant was measured by ELISA. *C*, Cytotoxic activities of pfp$^{-/-}$ CTL were tested in an 18 h [^{111}In]Ox release assay in the presence or absence of anti-FasL mAb (10 μg/ml). *D*, Cytotoxic activities of pfp$^{-/-}$ CTL against Renca pretreated with IFN-γ (500 U/ml) and TNF-α (400 U/ml) were tested in an 8 h [^{111}In]Ox release assay (E:T = 10 or 2:1) in the presence or absence of anti-LFA-1, anti-ICAM-1 mAbs (10 μg/ml), or the combination of anti-LFA-1 and anti-FasL mAbs. Data represent mean $\pm$ SD of triplicate samples. Similar results were obtained in three independent experiments.

Discussion

CD8$^+$ T cells have been demonstrated to be essential for the successful immunotherapy of the murine renal cancer Renca. This is

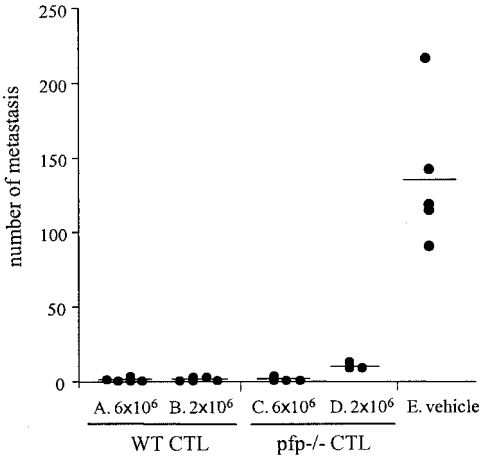

FIGURE 7. Adoptive transfer of pfp$^{-/-}$ CTL in 3-day experimental metastasis model. The mice were injected i.v. with Renca cells (1.5×10^5) on day 0. Three days later, CTL or vehicle were transferred to these tumor-bearing mice. WT CTL or pfp$^{-/-}$ CTL (6×10^6 or 2×10^6) were administered to WT BALB/c recipients. A or B vs E, $p < 0.01$; C or D vs E, $p < 0.05$.

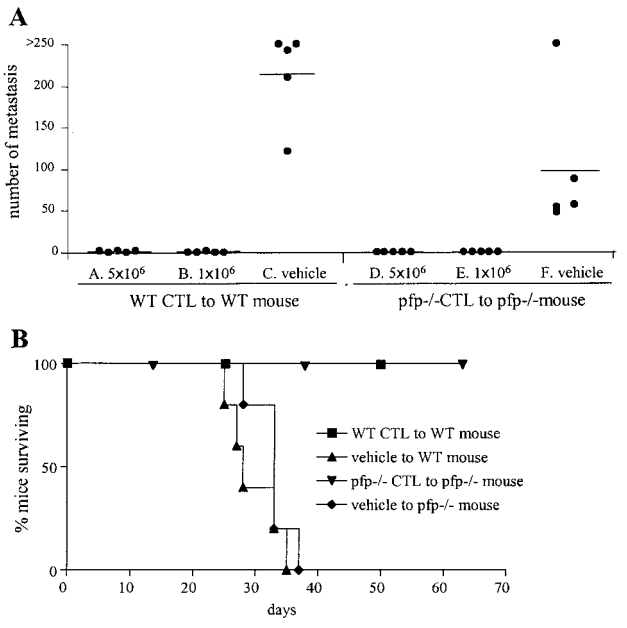

FIGURE 8. Adoptive transfer of CTL to pfp$^{-/-}$ mice. WT or pfp$^{-/-}$ mice were injected i.v. with 1.5×10^5 Renca cells on day 0. *A*, WT CTL or pfp$^{-/-}$ CTL (5×10^6 or 1×10^6) were administered to WT or pfp$^{-/-}$ BALB/c recipients, respectively. Lung metastases were quantified 17 days after i.v. injection of tumor cells. ●, A single mouse. A horizontal bar shows the average number of metastases in each group. The data shown are representative of two independent experiments. A or B vs C, $p < 0.01$; D or E vs F, $p < 0.01$; C vs F, $p = 0.09$. *B*, Survival of the remainder of the mice was monitored as shown.

the first study to describe the isolation and characterization of these tumor-specific CD8$^+$ T cells. These anti-Renca T cells can lyse Renca tumor cells in vitro by both the granule and FasL-mediated pathways. Interestingly, proinflammatory cytokines such as IFN-γ and TNF-α sensitize the tumor cells to lysis by both pathways, suggesting these cytokines could play an important role not only in the induction but also in the effector phase of the immune response. Sensitization of tumor cells to CTL-mediated lysis by increased expression of class I (26) or increased class I and ICAM-1 expression (27) has been reported in other experimental models. In our study, the induction of ICAM-1 and enhanced expression of Fas on the Renca target cells in response IFN-γ and TNF-α was instrumental for sensitizing these tumor cells to lysis by CTL (Fig. 1). Interaction between ICAM-1 on the tumor cells and LFA-1 on the CTL could stabilize the TCR interaction with Ag, thus, amplifying signals to the CTL. Granule-mediated lysis of Renca by CTL seems particularly dependent on ICAM-1/LFA-1 interactions (Fig. 3). This would be consistent with previous findings that a stronger signal was required to trigger degranulation as opposed to FasL-mediated lysis (28–32). Nonetheless, ICAM-1 expression on Renca also enhanced FasL-mediated lysis by 30–50% showing that ICAM-1/LFA-1 interaction can enhance tumor cell lysis via multiple killing pathways (Fig. 6).

The anti-Renca CTL generated in this study showed potent antitumor effects upon adoptive transfer to mice bearing established Renca pulmonary metastases. This finding allowed us to study the relative importance of different cytotoxic effector functions of CTL in vivo. This model allowed us to show that Renca lung metastases could be destroyed efficiently in the complete absence of perforin and granule-mediated cytotoxicity. These findings extend previous reports on clearance of lung metastases by CTL in a variety of murine tumors (33), as well as the murine melanoma model B16BL6-D5 (34), and the murine sarcomas MCA-310 (34) and MCA-205 (35). In the aforementioned studies, tumor-draining lymph node (TDLN) cells that had been expanded in vitro with anti-CD3 and IL-2 were used as a source of effector T cells. In contrast to our studies, relatively high numbers ($20–70 \times 10^6$) of these TDLN-derived CTL were required for successful adoptive immunotherapy of lung metastases, whereas as few as 1×10^6 anti-Renca CTL could provide complete regression of Renca pulmonary metastases. The reason for this quantitative difference in efficacy could reflect the way in which the respective CTL were generated. The short in vitro expansion of local TDNL cells (derived early in an immune response to tumor) with anti-CD3 and IL-2 could enhance the propagation of many T cells from the lymph node; however, the numbers of T cells in this population with functional antitumor effector capacity could be quite low. By contrast, our CTL were generated from mice which had rejected Renca after biological therapy, and had been rechallenged with tumor. Furthermore, T cells from the spleens of these mice were then expanded in vitro in the presence of irradiated tumor cells and IL-2. It would be anticipated that such a regimen would preferentially select for T cells with a high affinity for tumor. Nonetheless, despite quantitative differences in antitumor efficacy of the various CTL, our results in the Renca pulmonary metastases model are in close agreement to those previously reported using TDLN cells. That is, adoptive transfer of CTL derived from pfp$^{-/-}$ mice into pfp$^{-/-}$ mice bearing Renca pulmonary metastases resulted in complete tumor regression.

In the previous studies of Winter et al. (34, 36) and Peng et al. (35), it was also noted that TDLN-derived CTL from *gld* and IFN-γ $^{-/-}$ mice also displayed dramatic therapeutic benefits on administration to mice with pulmonary metastases, suggesting that neither FasL nor IFN-γ could account for the destruction of pulmonary metastases. However, in contrast to the tumors used in these studies, the Renca tumor we used is very sensitive to FasL-mediated lysis. We were unable to generate anti-Renca CTL from BALB/c *gld/gld* or IFN-γ $^{-/-}$ mice using our protocol, because these mice do not respond to in vivo immunotherapy (4). Interestingly, the transfer of WT BALB/c anti-Renca CTL plus neutralizing Ab to FasL did not block the antitumor effects in the pulmonary metastasis model (data not shown), suggesting FasL may

not be crucial for the destruction of Renca lung metastases. Nonetheless, with one cytotoxic pathway ablated, it is still possible that the second pathway could compensate, particularly in the Renca model where the tumor cells are very sensitive to both CTL lytic pathways. Future studies using CTL from mice deficient for both FasL and perforin will be required to determine the importance of these lytic pathways in antitumor responses to Renca in vivo. We are currently breeding these mice, and plan to generate CTL from them using various immunization protocols.

Although we observed no essential role for perforin in eradication of pulmonary Renca metastases by CTL, we cannot rule out the possibility that perforin could play an essential role in CTL-mediated rejection of Renca metastases in other tissue compartments. In the MCA-205 model, differences in perforin dependence were noted in the rejection of pulmonary or intracranial vs s.c. metastases (35). The rejection of s.c. tumor was perforin-dependent, whereas the rejection of pulmonary or intracranial metastases could proceed without an absolute requirement for perforin. Therefore, the relative importance of perforin for the therapeutic effects of CTL may depend on the anatomical location of the tumor. In a recent study on immunotherapy of the B16 melanoma, interesting differences in tumor rejection were observed depending on whether a tumor vaccine was applied in a prophylactic or therapeutic setting (37). In the prophylactic setting (several immunizations before tumor challenge), full protection was conferred in the absence of CD4, CD8, or NK 1.1^+ cells at the time of tumor challenge. Therefore, the prophylactic application of the vaccine allowed for the generation of antitumor responses that were not critically dependent on any one particular lymphocyte subset. By contrast, in the therapeutic setting (vaccine administered shortly after tumor challenge) depletion of $CD8^+$ and NK 1.1^+ cells hampered the rejection of small established tumors. Other studies in the B16 melanoma model using prophylactic vaccination showed that neither perforin nor FasL were important for the rejection of lung metastases (38). However, it should be noted that in the more clinically relevant therapeutic setting, perforin was essential for the rejection of the s.c. B16 tumors (37). $pfp^{-/-}$ mice did not respond to therapeutic vaccination of B16 cells with either tumor rejection or vitiligo, which can be a surrogate marker for an autoimmune response in this model. Thus, CTL may trigger multiple effector mechanisms to promote tumor destruction, and the dominant pathway(s) may vary depending on the anatomical location of the tumor.

The effects of adoptively transferred CD8 T cells in vivo could be diverse. In addition to direct killing of tumor cells, indirect effects of locally released cytokines on the tumor environment might be involved in tumor regression. Cytokines could induce local inflammation at tumor site and recruit other effector cells including macrophages, neutrophils, or NK cells. Macrophages and granulocytes have been observed infiltrating the lungs of B16BL6-D5 tumor-bearing mice after CTL adoptive immunotherapy (34). Furthermore, the production of cytokines or chemokines such as Mig or IP-10 at the tumor site could have antiangiogenic effects on the tumor vasculature (39–41). A recent study of IL-12 pulse IL-2 therapy in Renca model suggested a unique interrelationship between IFN-γ and the Fas/FasL pathway in mediating vascular endothelial apoptosis, inhibition of tumor neovascularization, and overall tumor regression (4). Furthermore, it was hypothesized that $CD8^+$ T cells were the most likely source of FasL. The expression of Fas by endothelial cell lines in vitro is enhanced by IFN-γ and TNF-α as is their sensitivity to FasL-mediated lysis (data not shown). Therefore, in a similar manner to direct Renca lysis, local cytokine production could sensitize endothelial cells to FasL-mediated "bystander" lysis by anti-Renca CTL. Whether specific anti-Renca CTL can promote concomitant vascular damage on infiltration of the tumor bed in vivo is of great interest, and is currently under investigation.

We have found no role for TRAIL in the direct lysis of Renca by CTL in vitro, despite the fact that Renca cells are known to be sensitive to the lytic effects of TRAIL (19, 25). TRAIL expression on human T cells is increased by type I IFNs, and this has been proposed to be a possible mechanism to explain the antitumor effects of IFNs in human renal cancer (42). In addition, TRAIL has also been implicated in the destruction of Renca liver metastases during IL-12 treatment, where liver NK cells seem to be the major source of TRAIL (24). Whether anti-Renca CTL play a direct or indirect role in the rejection of hepatic as well as peritoneal, lymphoid, or s.c. Renca metastases is unknown, and is currently under investigation. In conclusion, our results rule out a requirement for perforin in CTL-mediated tumor regression of pulmonary metastases in the Renca renal cancer. This begs the question as to exactly how T cells can destroy lung metastases of this tumor. The identification of molecular pathway(s) at different anatomical sites that are important for the CTL-mediated tumor destruction in vivo could provide important insights for the design of improved immunotherapeutic strategies for renal cancer in the future.

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