Targeting Lymphotoxin β Receptor with Tumor-Specific T Lymphocytes for Tumor Regression

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Abstract

Purpose: One of the impediments of immunotherapy against cancer is the suppression of tumor-specific CTLs in the tumor microenvironment, partly due to the selective inhibition of the perforin pathway and the emergence of Fas-resistant tumors. Therefore, we sought to identify perforin- and Fas-independent cytotoxic pathways and explored the potential of targeting LTβR with tumor-specific CTLs to induce tumor rejection *in vivo*.

Experimental Design: Fas-resistant tumors were examined for their susceptibility to perforin-deficient (pfp) CTLs via CTL adoptive transfer in mouse models of experimental lung metastasis. The specificity of LTβR, a cell surface death receptor, in causing tumor rejection by CTLs was analyzed by LTβR-specific neutralizing monoclonal antibody *in vitro*. The specificity and efficacy of LTβR in the suppression of established tumors was further investigated by silencing LTβR in tumor cells *in vivo*.

Results: pfp CTLs exhibited significant cytotoxicity against Fas-resistant tumors *in vivo*. The perforin- and Fas-independent cytotoxicity was directly mediated, at least in part, by the adoptively transferred CTLs. It was observed that LTβR was expressed on the tumor cell surface, and LTα, LTβ, and LIGHT, all of which are ligands for LTβR, were either constitutively expressed or activated in the tumor-specific CTLs and primary $CD8^+$ Tcells. Blocking LTβR with LTβR-specific neutralizing monoclonal antibody decreased CTL cytotoxicity *in vitro*. Silencing LTβR using LTβR-specific short hairpin RNA reduced the ability of pfp CTLs to induce tumor rejection *in vivo*.

Conclusion: LTβR directly mediates CTL-directed tumor rejection *in vivo*. Targeting LTβR with tumor-specific CTLs is a potential therapeutic approach.

CTLs lyse target tumor cells through two primary cellular effector mechanisms (1). The first cytolytic pathway depends on the polarized secretion of perforin and granzymes. The second effector mechanism involves the interaction of FasL on the activated CTL with its receptor Fas on the target cell surface (2–6). Despite the fact that the perforin pathway is the dominant antitumor effector mechanism (4, 5, 7, 8), recent studies have begun to shed light on the importance of other cytotoxic effector mechanisms of tumor-specific CTLs for the suppression of tumor growth (9, 10). For example, Caldwell et al. (6) observed that the perforin pathway of tumor-specific CTLs mediated strong antitumor effects in a minimal disease setting, but that both the perforin and FasL-dependent effector mechanisms were essential for optimal tumor regression under conditions of extensive tumor burden. Seki et al. (3) have also observed that although perforin-mediated killing was of paramount importance for CTL-mediated lysis *in vitro*, some *in vivo* effector mechanisms were clearly independent of perforin as illustrated in a Renca pulmonary metastasis model. Dobrzanski et al. (11) reported that although tumor cytolysis was predominantly perforin-dependent *in vitro*, the therapeutic effects of CTL-based immunotherapy were dependent, in part, on effector cell–derived LTα in a B16 lung metastasis model. Furthermore, it has been shown that both D122 Lewis lung carcinoma and melanoma were rejected by tumor-specific CTLs through a cytolytic mechanism that was independent of both perforin and Fas pathways *in vivo* (12, 13). These studies suggest that other cytotoxic effector pathways, in addition to perforin and Fas/FasL, play significant roles in the inhibition of tumor growth.

LTβR is a member of the tumor necrosis factor receptor (TNFR) superfamily, and was initially identified as a critical mediator controlling the development and organization of the secondary lymphoid tissues (14). However, it is increasingly appreciated that the LTβR signaling pathway is involved in numerous other biological processes, including the initiation of extrinsic apoptotic cell death in tumor cells (15–19). Engagement of LTβR with agonistic anti-LTβR monoclonal antibody (mAb), or recombinant ligand proteins (LTα1LTβ2 or LIGHT) induced the cell death of several types of tumor cells (15–19). Moreover, Lukashev et al. recently examined LTβR protein in clinical human tumor tissues and observed that 87% to 96% of colorectal, lung, larynx/pharynx, stomach, and melanoma tumors were LTβR-positive (19). These authors further showed

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that anti-LTβR agonistic mAb effectively inhibited human colorectal tumor growth in xenograft mouse models (19). In this study, we revealed that LTβR directly mediates a CTL-directed perforin- and Fas-independent cytotoxic effector mechanism *in vivo*. We further showed that targeting LTβR with tumor-specific CTLs via adoptive transfer is a potential therapeutic approach in the induction of tumor rejection *in vivo*.

Materials and Methods

Mice. Female BALB/c (H-2^d) mice were obtained from Charles River Laboratories. Female perforin-deficient (pfp) mice on a BALB/c background were kindly provided by M. Smyth (Peter MacCallum Cancer Institute, East Melbourne, Australia) via R. Wiltrout (LEI, CCR, National Cancer Institute. Frederick, MD). All mice were housed, maintained, and studied in accordance with the approved guidelines of the NIH and Medical College of Georgia for animal use and handling.

Tumor cells. The CMS4 sarcoma line was kindly provided by A. Deleo (University of Pittsburgh, Pittsburgh, PA). The CMS4-met subline was produced from the parental CMS4 population by one *in vivo* passage in the lungs of normal BALB/c mice, as described (20, 21). The mammary carcinoma cell line, 4T1, was obtained from the American Type Culture Collection.

Cell surface marker analysis. Tumor cells were immunostained with fluorescent-conjugated anti-Fas mAb (PharMingen) or an isotype-matched hamster IgG, and analyzed by flow cytometry. For IFN-γR, LTβR and TNFαR staining, tumor cells were incubated with biotin anti-mouse IFNγR (PharMingen), anti-mouse LTβR (eBiosciences) or anti-mouse TNFα (PharMingen) mAbs, followed by incubation with Streptavidin Tricolor conjugate (CALTAG) or FITC-conjugated anti-hamster IgG (Kirkegaard & Perry Laboratories). The stained cells were analyzed with flow cytometry.

Measurement of Fas-mediated cell death. Cell death was measured by propidium iodide (PI) staining as described previously (22). Tumor cells were treated with recombinant IFN-γ (100 units/mL; R&D Systems) or TNFα (100 units/mL; R&D Systems), or both, overnight. The cytokine-treated cells were incubated with recombinant human FasL (100 ng/mL, PeproTech) for ~22 h. Cells were then analyzed by PI staining and flow cytometry. The percentage of cell death was calculated by the formula: (% PI^+ cells with sFasL) - (% PI^+ cells without sFasL).

Production of tumor-specific $CD8^+$ CTL lines. $CD8^+$ CTL lines reactive against the CMS4 sarcoma were established from either wild-type (wt) BALB/c mice, BALB/c-gld mice, or BALB/c-pfp mice using an anti–CTLA-4 mAb–based immunotherapy, as previously described (6). Spleen-derived $CD8^+$ CTL lines were maintained and propagated in 24-well plates (2×10^5/well) by weekly stimulation with irradiated (20 Gy) syngeneic normal BALB/c splenocytes (5×10^6/well) as APC and irradiated (200 Gy) CMS4-met cells (1×10^5/well) as a source of cognate antigen and IL-2 (60 IU/mL; Hoffmann-La Roche).

Cytotoxicity assay. CTL activity was assessed by ^{51}Cr release assays as previously described (23). To analyze the role of LTβR, lytic assays were done in the absence or presence of anti-LTβR mAb (clone AC.H6; PharMingen). ^{51}Cr-labeled CMS4-met.vFLIP cells (1×10^4 cells) were preincubated with anti-LTβR mAb (10 μg/mL) in 100 μL culture medium in 96-well, U-bottomed plates at 4°C for 30 min. pfp CTLs (1×10^5 cells in 100 μL medium) were then added, and cultured for ~18 h. The percentage of specific ^{51}Cr release was calculated according to the following formula: % lysis = (experimental cpm - spontaneous cpm) / total cpm × 100%.

CTL adoptive transfer. Treatment of tumor-bearing mice by CTL adoptive transfer was conducted as previously described (23). To inactivate endogenous host immune cells, mice were irradiated in a Gammacell 40 Exactor Radiator (Nordion International) for a total dose of 5 Gy. Four hours later, tumor cells were injected into mice as described above.

Reverse transcription-PCR analysis. Total RNA was isolated from tumor cells using RNA STAT-60 reagent (Tel-Test) according to the manufacturer's instruction, and used for first strand cDNA synthesis using the ThermoScript reverse transcription-PCR (RT-PCR) system (Invitrogen). The cDNA was then used as templates for PCR amplification. The sequences of the primers used are listed in Table 1.

Stable transfection of tumor cells. CMS4-met and 4T1 cells were transfected with the mammalian expression plasmid pEGFPN1 (Invitrogen) containing the *vFLIP* gene. Transfections were done using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions. The transfected cells were propagated and maintained in culture medium containing geniticin (0.75 mg/mL; Invitrogen).

Stable short hairpin RNA expression plasmid construction. To produce stable short hairpin RNA (shRNA) expression constructs, two complimentary pairs of oligonucleotides encoding a 21-bp mLTβR-specific double-stranded siRNA (5′-GGCACAGAAGCCGAGGTCACA-3′) and a 20-bp scramble double-stranded siRNA (5′-ATAGCGACTAAACA-CATCAA-3′) were synthesized and cloned to psiRNA-h7SKgzGFP expression vector (Invivogen), respectively, according to the manufacturer's instructions. The resulting expression constructs express a 49 and a 48 bp RNA that forms a double-stranded RNA with a hairpin structure, termed shRNA. The shRNA expression vectors containing scramble shRNA and mLTβR-specific shRNA were transfected into CMS4-met.vFLIP cells using LipofectAMINE 2000 according to the manufacturer's instructions. Stable transfectants were selected by using Zeocin. The transfectants were further selected by cell sorting based on the GFP intensity in a cell sorter (Dako MoFlo).

Results

Tumor-specific CTLs execute antitumor cytotoxicity through perforin- and Fas-independent effector mechanisms. CMS4-met tumor cells were stably transfected with a mammalian expression vector pEGFPN1 expressing Fas-mediated apoptosis

Table 1. PCR primer sequences used in this study

	Forward	Reverse
Fas L	5′-CTTGGGCTCCTCCAGGGTCAGT-3′	5′-TCTCCTCCATTAGCACCAGATCC-3′
Granzyme A	5′-CCTGAAGGAGGCTGTGAAAGAATC-3′	5′-CCTGCTACTCGGCATCTGGTTC-3′
Granzyme B	5′-GCCCACAACATCAAAGAACAGG-3′	5′-CCAGAATCCCCCCGAAAGG-3′
gp70	5′-ACCTTGTCCGAAGTGACCG-3′	5′-GTACCAATCCTGTGTGGTCG-3′
IFNγ	5′-ATGGCTGTTTCTGGCTGTTACTG-3′	5′-GCTTCCTGAGGCTGGATTCC-3′
LTα	5′-TGCCAGGACAGCCCATCCAC-3′	5′-TGAGCAGGAACACAGCCCC-3′
LTβ	5′-TGGATGACAGCAAACCGTCG-3′	5′-AACGCTTCTTCTTGGCTCGC-3′
LIGHT	5′-GGCTGGAACAGAACCACCG-3′	5′-CCAAGTCGTGTCTCCCATAACAGAG-3′
Perforin	5′-CCACAGGCTCATCTCCTCCTATG-3′	5′-TCCACCAGACCAGGGTTGC-3′
TNFα	5′-TGACAAGCCTGTAGCCCACG-3′	5′-GACTCCAAAGTAGACCTGCCCG-3′

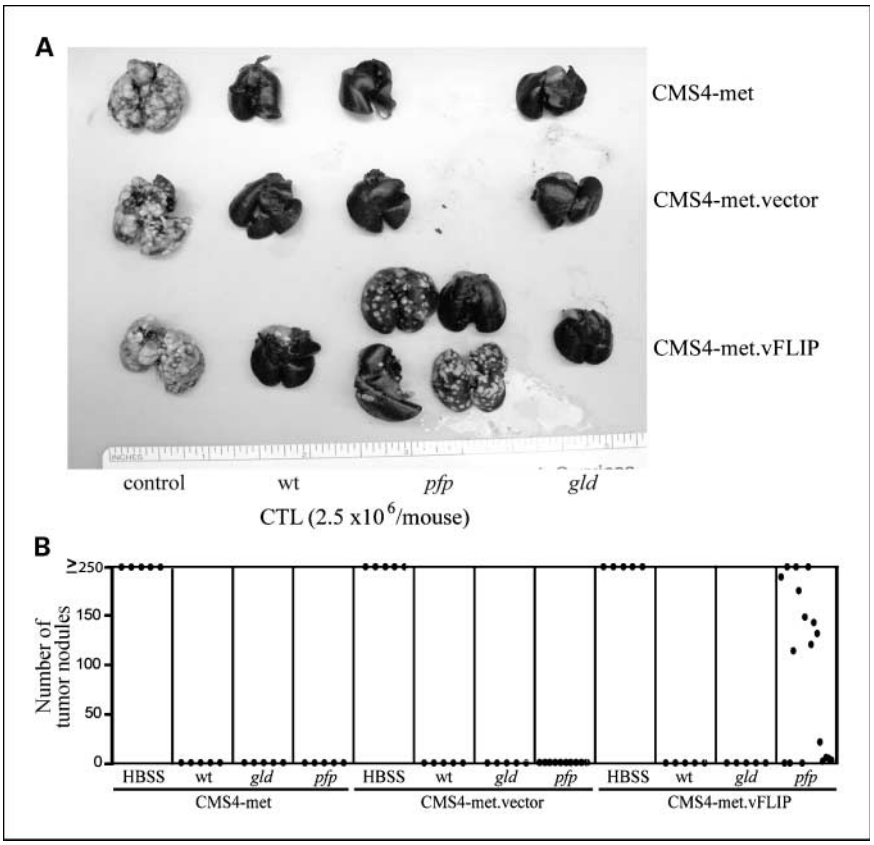

Fig. 1. Fas-resistant tumor cells are susceptible to pfp CTL-mediated cytotoxicity. Susceptibility of Fas-resistant CMS4-met.vFLIP tumor cells to wt, pfp, and gld CTLs in an adoptive immunotherapy mouse model. CMS4-met, CMS4-met.vector, and CMS4-met.vFLIP cells (2.5×10^5 cells/mouse) were injected i.v. into the tail veins of naïve, immunocompetent BALB/c mice. Three days later, wt, pfp, and gld CTL (2.5×10^6 /mouse) or HBSS saline (as control) were injected i.v. into the tail veins of tumor-bearing mice. Mice were euthanized 14 d after adoptive CTL transfer for enumeration of lung tumor nodules. *A,* images of lungs from representative mice receiving various tumor cells and CTLs. *B,* the number of lung tumor nodules was enumerated in a single blinded fashion. Dots, total counts from independent mice.

inhibitor vFLIP. Nontransfected (CMS4-met), vector-transfected (CMS4-met.vector), and vFLIP-transfected (CMS4-met.vFLIP) tumor cells were injected i.v. into naïve mice to establish experimental lung metastasis. Three days later, tumor-specific wt, pfp, and gld CTLs were adoptively transferred i.v. into the tumor-bearing mice. Examination of the lungs revealed that wt and gld CTL effectively suppressed all detectable tumor growth (Fig. 1). pfp CTLs also completely inhibited CMS4-met and CMS4-met.vector tumor growth because of their Fas sensitivity (Fig. 1), as previously reported (23). Interestingly, pfp CTLs also significantly, but incompletely, inhibited CMS4-met.vFLIP tumor growth (Fig. 1). Because CMS4-met.vFLIP cells are completely resistant to Fas-mediated apoptosis (Fig. 2B), pfp CTLs should not reject CMS4-met.vFLIP cells if perforin and Fas-mediated cytotoxicity are the only two cytotoxic effector mechanisms. The inhibition of CMS4-met.vFLIP cells by pfp CTLs suggests the existence of a perforin- and Fas-independent cytotoxic pathway that functions *in vivo*.

To exclude the possibility that pfp CTL-mediated suppression of CMS4-met.vFLIP tumors *in vivo* was due to loss of vFLIP expression in tumor cells *in vivo*, we injected CMS4-met.vFLIP tumor cells into mice and recovered tumor cells from mouse lungs 17 days after tumor implantation. The recovered cells were analyzed for GFP expression (GFP exists as a fusion protein with vFLIP and is thereby a surrogate indicator of the presence of the expression vector in the cells) and sensitivity to Fas-mediated apoptosis. CMS4-met.vFLIP cells recovered from three separate mice all maintained GFP expression (Fig. 2A) and were still resistant to Fas-mediated apoptosis (Fig. 2B). Therefore, we concluded that vFLIP is stably expressed in the transfected tumor cells *in vivo*, and that the cells retained resistance to Fas-mediated apoptosis.

The pfp CTLs were generated from perforin-knockout mice and are thus completely negative for wt perforin. To ensure that the pfp CTLs have no wt CTL contamination, we analyzed perforin expression by RT-PCR analysis in wt and pfp CTLs. The pfp mouse contains a DNA fragment insertion in its perforin coding sequence. We designed a pair of PCR primers to cover the coding region containing the insertion. RT-PCR analysis indicated, as expected, that the pfp CTLs contained no wt perforin (Fig. 2C). Therefore, we concluded that no wt CTL contamination contributed to the lysis of Fas-resistant CMS4-met.vFLIP tumor cells by pfp CTLs.

Tumor-specific pfp CTLs are directly responsible for rejection of Fas-resistant tumors. In this experimental lung metastasis model, the experiment was completed in 17 days. Therefore, it is unlikely that endogenous T lymphocytes were responsible for the tumor rejection response. However, to preclude the likelihood that tumor rejection was mediated by host immune cells, recipient mice were sublethally irradiated prior to the implantation of CMS4-met.vFLIP cells and the adoptive transfer of pfp CTLs. The efficacy of pfp CTLs in the rejection of Fas-resistant tumors was then examined in these irradiated mice.

The irradiated mice died more quickly than the nonirradiated mice after CMS4-met.vFLIP implantation (Fig. 2D), suggesting that the host immune cells, probably the innate immune cells, play a role in tumor suppression. Adoptive transfer of pfp CTLs effectively inhibited CMS4-met.vFLIP tumor growth in the lung, and the degree of inhibition was even greater in irradiated mice than in nonirradiated control mice (Fig. 2E), suggesting

that (*a*) the tumor-specific pfp CTLs possess perforin- and Fas-independent cytotoxic effector mechanisms, and that (*b*) radiation might eliminate immunosuppressive cells (24, 25) in the host and thus enhance pfp CTL-mediated cytotoxicity against Fas-resistant CMS4-met.vFLIP cells.

***IFN-γ and TNFα do not induce direct tumor cell death* in vitro.** Tumor-specific T cells secrete abundant amounts of IFN-γ and TNFα after interaction with antigen-bearing tumors. To determine whether IFN-γ or TNFα play a direct role in the death of Fas-resistant CMS4-met.vFLIP cells, we examined the sensitivity of these Fas-resistant tumor cells to IFN-γ or TNFα *in vitro*. RT-PCR analysis indicated that stimulation of pfp CTLs by tumor cells rapidly induced the expression of both IFN-γ and TNFα (Fig. 3A). Immunostaining of tumor cells with mAb that are specific for IFN-γR and TNFαR revealed that both IFN-γR and TNFαR are expressed on the tumor cell surfaces (Fig. 3A). Furthermore, exposure of CMS4-met.vFLIP cells to TNFα or IFN-γ up-regulated Fas, a gene known to be activated by IFN-γ and TNFα, indicating that TNFαR and IFN-γR are functionally responsive (Fig. 3B). However, treatment with TNFα or IFN-γ or with both TNFα and IFN-γ did not induce any detectable cell death in CMS4-met.vFLIP cells *in vitro* (Fig. 3C and D), suggesting that these soluble cytokines might not be the direct cause of pfp CTL-mediated cytotoxicity against CMS4-met.vFLIP cells.

Tumor-specific pfp CTLs suppress Fas-resistant mammary carcinoma tumor growth. To determine whether the inhibition of Fas-resistant tumor growth by pfp CTLs is tumor type–specific, we sought to extend our findings to another type of tumor. Because these tumor-specific CTLs are H-2L^d-restricted and recognizes an epitope mapped to the MuLV gp70 protein (20), we screened various tumor cell lines and identified a mammary carcinoma cell line, 4T1, that expresses both gp70 (antigen) and H-2L^d (Fig. 4A). Next, we transfected 4T1 cells with the empty vector (4T1.vector) or the vector containing vFLIP (4T1.vFLIP) and established stable sublines. Interestingly, although 4T1.vector cells were poorly Fas-sensitive *in vitro* (<10% cell death; data not shown) and the vFLIP-transfected cells were completely Fas-resistant, somewhat different results were observed *in vivo*, presumably due to the longer-term interactions *in vivo*. The stable transfectants were injected into syngeneic mice, followed by adoptive transfer of pfp CTLs. Examination of tumor growth in the lung indicated that the pfp CTLs exhibited significant cytotoxicity against 4T1.vector cells (Fig. 4B). However, the Fas-resistant 4T1.vFLIP cells also exhibited significant susceptibility to pfp CTLs, but not to the same level as seen with the vector control (Fig. 4B). Thus, these observations indicate that pfp CTLs could elicit cytotoxicity against Fas-resistant mammary tumors through a perforin- and Fas-independent effector mechanism.

Expression of LTβR in tumor cells and activation of LTβR ligands in T cells. The above results strongly suggest that these tumor-specific CTLs mediate an additional cell contact–dependent cytotoxic pathway. In the literature, it has been shown that, like Fas, LTβR is a death receptor that mediates apoptosis in different types of tumor cells (14–19). Therefore, we hypothesized that LTβR might be responsible for the perforin- and Fas-independent effector mechanism elicited by these tumor-specific CTLs. To test this hypothesis, we first analyzed LTβR expression in CMS4-met and 4T1 tumor cells. Staining the cell surface of LTβR with LTβR-specific mAbs indicated that LTβR is expressed in both tumors (Fig. 5A). Next, we analyzed the expression of LTα, LTβ, and LIGHT, all of which are ligands for LTβR, during CTL activation. We also analyzed the expression of key molecules in the perforin and Fas pathways during CTL activation. RT-PCR analysis revealed

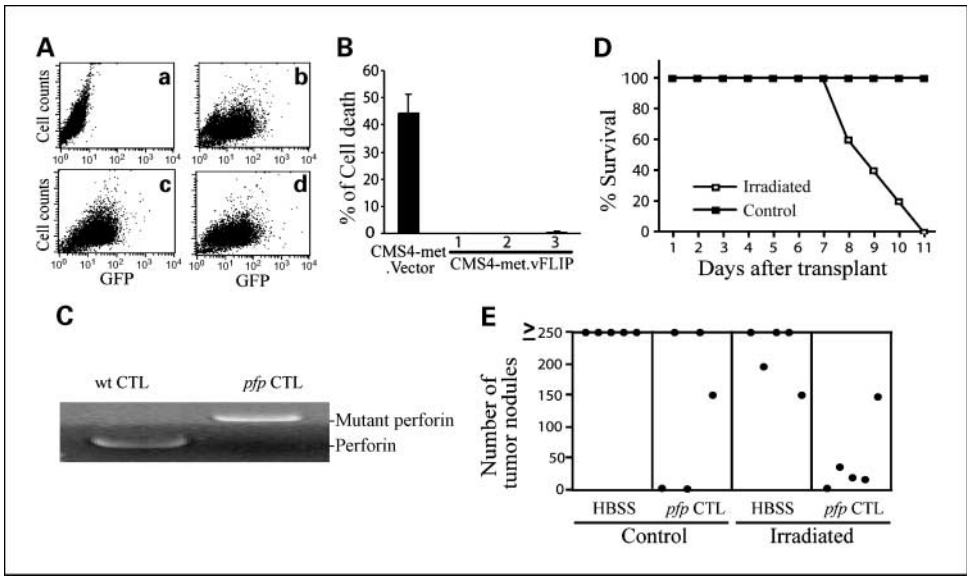

Fig. 2. Lysis of Fas-resistant tumor cells is directly mediated by adoptively transferred pfp CTLs. *A,* GFP intensity of CMS4-met cells (*a*) and CMS4-met.vFLIP cells recovered from three mice (*b-d*). *B,* apoptosis assay of CMS4-met.vector and CMS4-met.vFLIP cells recovered from the three mice described in (*A*). Cells were preincubated with IFN-γ and TNFα, followed by culture with sFasL (100 ng/mL). Cell death was measured by PI staining and analyzed by flow cytometry. Columns, mean of three separate experiments; bars, SD. *C,* RT-PCR analysis of perforin expression in wt and pfp CTLs. The PCR primers were designed to cover the disrupted (insertion) region of the perforin cDNA. The amplified wt perforin and mutant perforin are indicated. *D,* naïve BALB/c mice were sublethally irradiated (5 Gy) to inactivate host immune cells. CMS4-met.vFLIP cells (2.5×10^5 cells/mouse) were injected i.v. into nonirradiated (control) and irradiated mice. Survival was recorded. *E,* CMS4-met.vFLIP cells (2.5×10^5 cells/mouse) were injected i.v. into nonirradiated (control) and irradiated mice. Three days later, pfp CTLs (1×10^6/mouse) or saline (HBSS) were also injected i.v. into tumor-bearing mice. Mice were euthanized 14 d after adoptive CTL transfer. The number of lung tumor nodules was enumerated. Dots, counts from independent mice.

that three key molecules in the perforin pathway: perforin, granzyme A, and granzyme B, were all up-regulated in activated CTLs, as did FasL, the ligand for the Fas receptor (Fig. 5B). LTβ was constitutively expressed, whereas LTα and LIGHT were up-regulated in the activated CTLs (Fig. 5B). Therefore, it is clear that the key molecules involved in these three pathways were coordinately activated during CTL activation.

Because the CTLs are established T cell lines, we purified primary $CD8^+$ T cells from naïve mice and stimulated them with anti-CD3 and CD28 mAb. RT-PCR analysis of LTα, LTβ, and LIGHT expression indicated that the activation kinetics of these ligands were very similar to that of the established CTLs (Fig. 5C). Therefore, we concluded that LTα, LTβ, and LIGHT activation is associated with T cell activation and is a general phenomenon.

Tumor-specific CTLs execute antitumor cytotoxicity through LTβR. Our above findings indicate that LTβR is expressed on the tumor cell surface and all ligands for LTβR are either constitutively expressed or activated during T cell activation, suggesting that the LTβR pathway might be an effector pathway that mediates the destruction of Fas-resistant tumor cells by pfp CTLs. To test this hypothesis, we first sought to block the function of LTβR on the tumor cell surface using a LTβR-specific neutralizing mAb (26). The Fas-resistant tumor cells were incubated with the neutralizing antibody and then coincubated with pfp CTLs in an *in vitro* CTL assay. Measurement of cell death revealed that blocking LTβR on the tumor cell surface significantly decreased tumor cell sensitivity to CTL-mediated cytotoxicity ($P = 0.007$; Fig. 5D).

The above mAb neutralization experiments indicate that LTβR directly mediates CTL killing. To further show that function, we used a second approach. We constructed a stable shRNA expression vector that constitutively expresses a shRNA specific for mouse LTβR. Expression of LTβR-specific shRNA significantly ($P = 0.002$) decreased LTβR expression on the

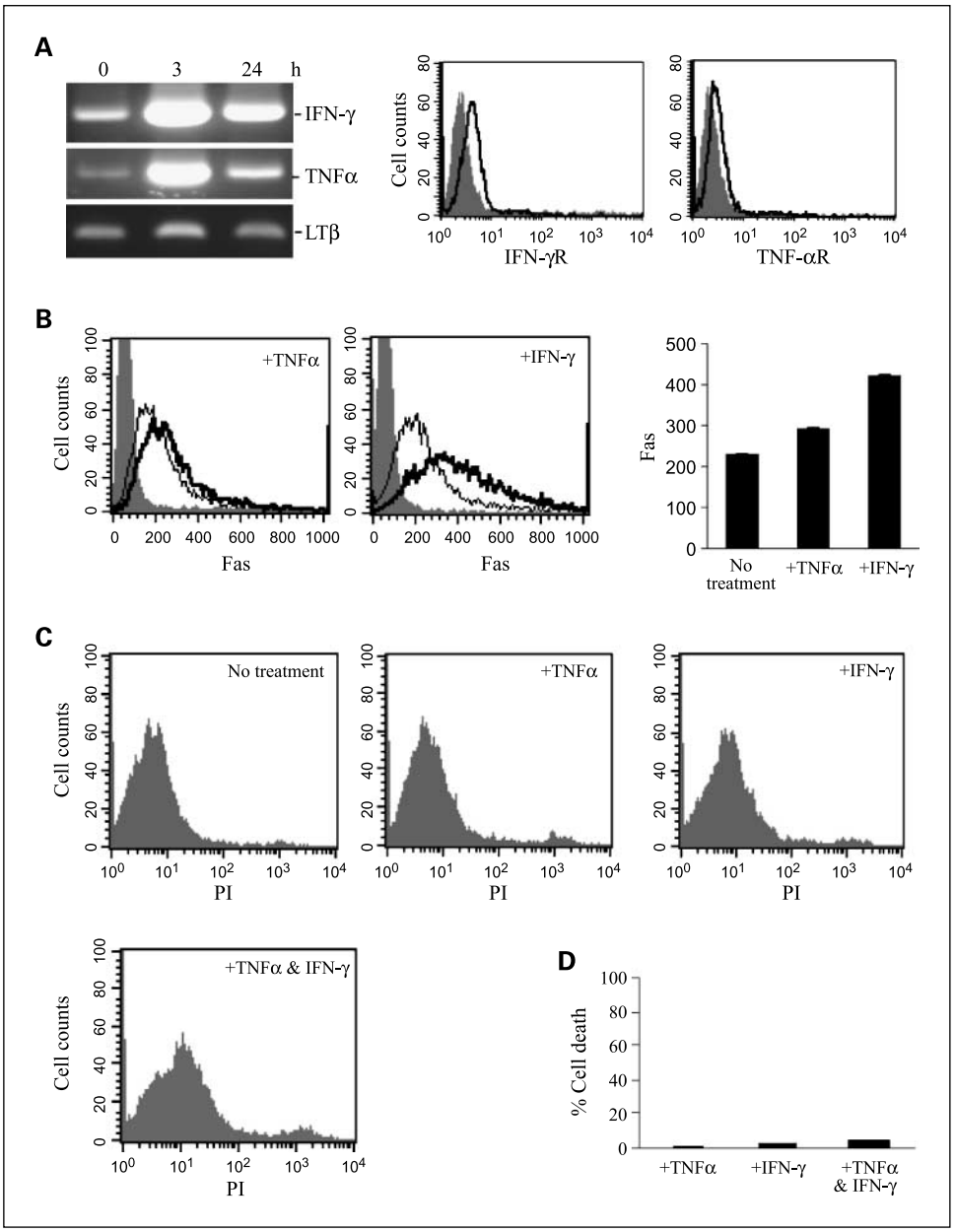

Fig. 3. IFN-γ and TNFα elicited no direct antitumor activity. *A,* expression of IFN-γ and TNFα in pfp CTLs and IFN-γR and TNFαR in CMS4-met.vFLIP cells. pfp CTLs were stimulated with irradiated CMS4-met cells for 3 or 24 h and analyzed for IFN-γ and TNFα transcript levels by RT-PCR analysis (*left*). LTβ is used as normalization control. For IFN-γR and TNFαR analysis, tumor cells were stained with IFN-γR or TNFαR-specific mAbs, respectively, and analyzed by flow cytometry (*middle* and *right*). *B,* responsiveness of CMS4-met.vFLIP cells to IFN-γ and TNFα. Tumor cells were treated with IFN-γ or TNFα for ~22 h and analyzed for activation of Fas by immunostaining with Fas-specific mAb (*left* and *middle*). Fas expression levels in untreated cells (*thin line*) and cytokine-treated cells (*thick line*). Staining with isotype control mAb (*shaded area*). Right, mean fluorescent intensity of Fas (*left* and *middle*). Data are expressed as mean ± SD of three separate experiments. Columns, means; bars, SD. *C,* sensitivity of CMS4-met.vFLIP cells to IFN-γ (+*IFN-γ*) or TNFα (+*TNFα*), or both IFN-γ and TNFα (+*IFN-γ & TNFα*). Tumor cells were treated with IFN-γ, TNFα, or both IFN-γ and TNFα for ~22 h and analyzed for cell death by PI staining and flow cytometry. Histograms of PI staining of cytokine-exposed cells. *D,* percentage of cell death. Data are expressed as mean ± SD of three separate experiments. Column, mean; bars, SD.

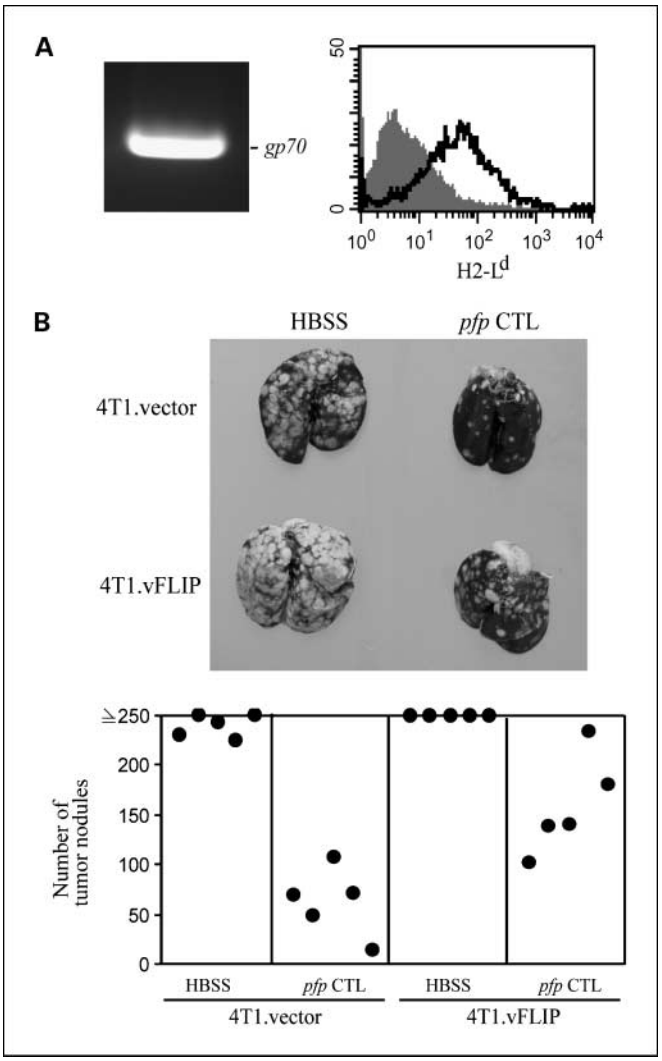

Fig. 4. Fas-resistant 4T1 mammary carcinoma cells are susceptible to pfp CTL-mediated cytotoxicity. *A,* antigen and MHC class I expression in the 4T1 mammary carcinoma cell line. Left, RT-PCR analysis of gp70 transcript level. Right, analysis of cell surface MHC class I H-2L^d levels by flow cytometry. H-2L^d-specific staining (*solid line*). Staining with mAb isotype control (*shaded area*). *B,* susceptibility of Fas-resistant 4T1.vFLIP tumor cells to pfp CTLs in an adoptive immunotherapy mouse model. 4T1.vector and 4T1.vFLIP cells (2.5×10^5 cells/mouse) were injected i.v. into BALB/c mice. Three days later, pfp CTLs (3×10^6/mouse) or HBSS saline (as control) were injected i.v. into tumor-bearing mice. Mice were euthanized 14 d after adoptive CTL transfer. Top, images of representative mouse lungs. Bottom, the number of lung tumor nodules was enumerated. Dots, total counts from independent mice.

tumor cell surface (Fig. 6A) and silencing LTβR did not alter tumor cell ability to colonize and grow in the lungs (Fig. 6B). It is important to point out that a lower dose of tumor cells (1×10^5 cells/mouse) was used in this experiment to allow quantitative comparison of tumor nodule number between mice that received the various tumor sublines without CTL adoptive transfer.

To determine whether LTβR mediates CTL killing, we injected mice with tumor sublines that constitutively express either a scramble shRNA (CMS4-met.vFLIP.psiRNA.scramble) or LTβR-specific shRNA (CMS4-met.vFLIP.psiRNA.LTβR) sequence. The pfp CTLs were then adoptively transferred to the tumor-bearing mice. It is important to point out here that a higher concentration of pfp CTL (3×10^6/mouse) was used (Fig. 6C) as compared with Figs. 1 and 2 (2.5×10^6 and 1×10^6cells/mouse, respectively) in an effort to reduce the variability in the observed antitumor response and to better unmask the potential contribution of an alternative CTL-mediated tumor rejection mechanism. At this higher pfp CTL concentration, we observed stronger antitumor effects against the Fas-resistant tumors (Fig. 6C). More importantly, the CMS4-met.vFLIP.psiRNA.LTβR tumor cells became significantly less susceptible to pfp CTLs as compared with CMS4-met.vFLIP.psiRNA.scramble tumor cells ($P = 0.001$; Fig. 6C). Therefore, we concluded that the pfp CTLs lyse the Fas-resistant CMS4-met.vFLIP tumor cells through a LTβR-mediated effector mechanism.

Discussion

The TNFR superfamily consists of at least 28 receptors and 18 ligands (27). Many of the members in this superfamily, including TNFα, LTα, LIGHT, and TRAIL, have been well

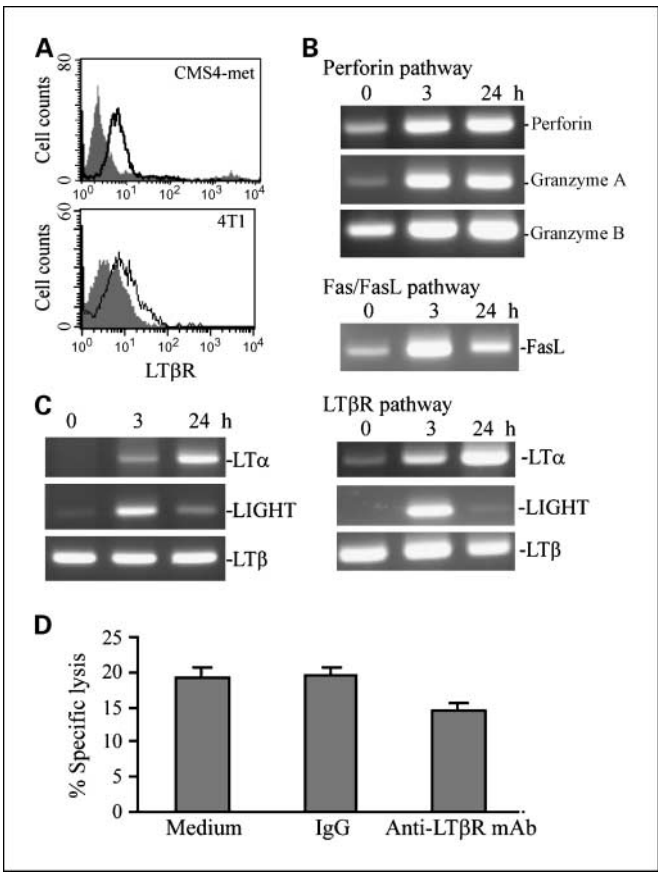

Fig. 5. Expression of LTβR and its ligands in tumor cells and T lymphocytes and function of LTβR in CTL-mediated antitumor cytotoxicity. *A,* LTβR expression on tumor cell surface. CMS4-met and 4T1 tumor cells were stained with LTβR-specific mAb and analyzed by flow cytometry. LTβR-specific staining (*solid lines*) and staining with mAb isotype control (*shaded area*). *B,* RT-PCR analysis of the expression kinetics of key molecules of the perforin, Fas, and LTβR-mediated effector pathways in tumor-specific CTLs. Tumor-specific CTLs were stimulated with irradiated tumor cells and harvested for RT-PCR analysis of perforin, granzyme A, granzyme B, Fas L, LTα, LTβ, and LIGHT. *C,* RT-PCR analysis of LTα, LTβ, and LIGHT in mouse primary CD8$^+$ T cells. CD8$^+$ T cells were isolated from naïve mouse spleen and stimulated with anti-CD3 and CD28 mAb for 3 or 24 h. *D,* blocking LTβR function decreased tumor cell susceptibility to CTL-directed cytotoxicity. CMS4-met.vFLIP cells were analyzed for their susceptibility to pfp CTLs in the presence of neutralizing anti-LTβR mAb. Medium control (*medium*) and isotype-matched IgG (*IgG*) were used as controls. Columns, means; bars, SD.

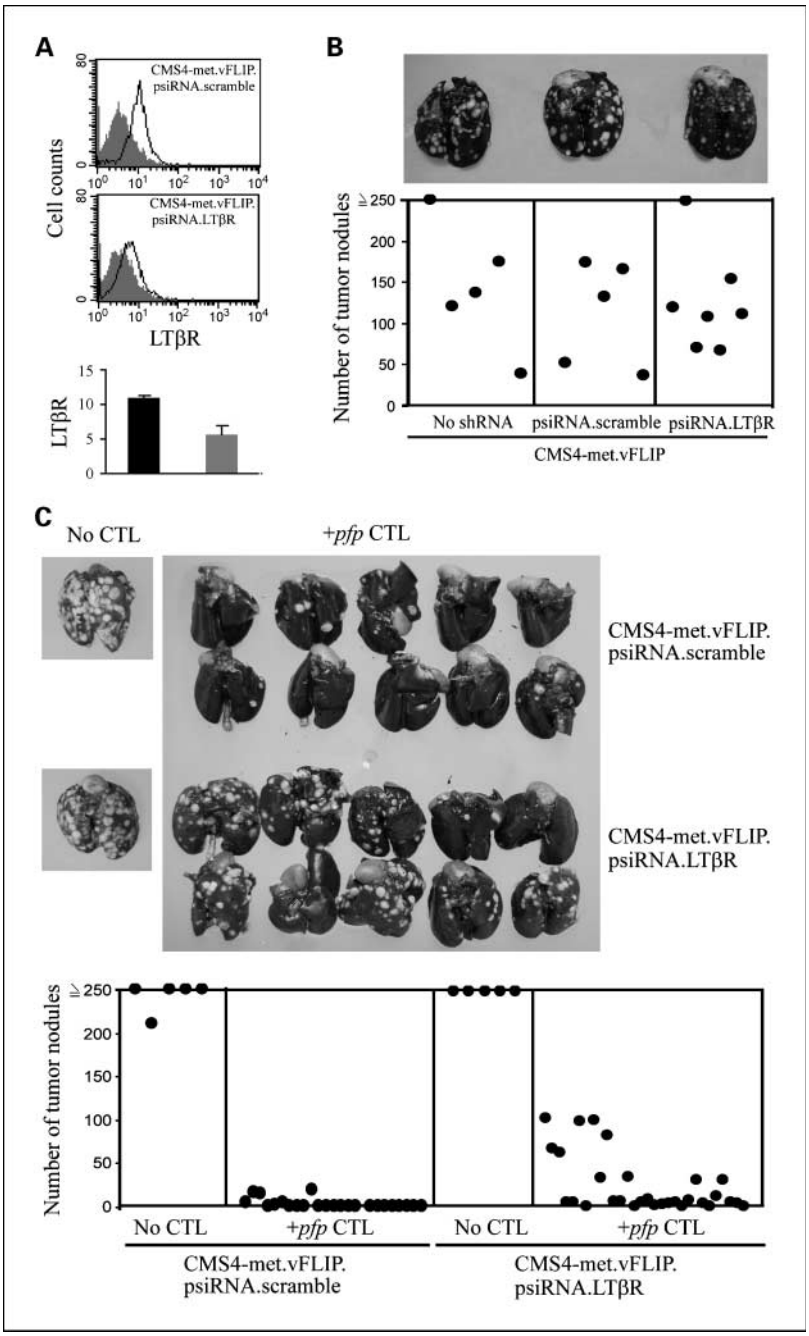

Fig. 6. Silencing LTβR reduced the susceptibility of Fas-resistant tumor cells to pfp CTL-mediated tumor rejection. *A,* constitutive expression of LTβR-specific shRNA diminished LTβR expression levels in CMS4-met.vFLIP cells. CMS4-met.vFLIP cells were transfected with psiRNA-gz-h7SK expression vectors containing a scramble shRNA coding sequence (*CMS4-met.vFLIP.psiRNA.scramble*) or LTβR shRNA – specific coding sequence (*CMS4-met.vFLIP.psiRNA.LTβR*). The tumor cells were stained with LTβR-specific mAbs and analyzed by flow cytometry. LTβR-specific staining (*solid line*) and mAb isotype control (*shaded area*). Bottom, mean fluorescence intensity of LTβR (*top* and *middle*). Data are expressed as mean ± SD of three separate experiments. Columns, means; bars, SD. Black column, LTβR mean fluorescence intensity of CMS4-met.vFLIP.psiRNA.scramble cells; gray column, LTβR mean fluorescence intensity of CMS4-met.vFLIP.psiRNA.LTβR cells. *B,* silencing LTβR did not alter tumor cell metastatic potential. CMS4-met.vFLIP and shRNA-expressing cells (1×10^5 cells/mouse) were injected i.v. into naïve mice. Mice were euthanized 14 d later. Top, images of representative lungs from mice receiving various tumor cells. Bottom, the number of lung tumor nodules was enumerated. *C,* silencing LTβR decreased the susceptibility of Fas-resistant tumor cells to pfp CTL-mediated cytotoxicity. CMS4-met.vFLIP cells stably expressing scramble and LTβR-specific shRNA (2.5×10^5 cells/mouse) were injected i.v. into BALB/c mice. Three days later, pfp CTLs (3×10^6/mouse) or HBSS saline (no CTLs, as control) were injected i.v. into tumor-bearing mice. Mice were euthanized 14 d after adoptive CTL transfer. Top, images of lungs from mice receiving various tumor cells and CTLs (one of three independent experiments). Bottom, the number of lung tumor nodules was enumerated. Data are from three independent experiments.

documented to be involved in tumor cell apoptosis (28–32). The LTβR was initially identified to be critical for the organization of lymphoid tissues, lymph nodes, and Peyer patches during embryogenesis and development, and maintenance of secondary lymphoid architectures in adults (33–36). However, it has been well established that the LTβR signaling pathway is involved in the initiation of apoptotic death in tumor cells (15–19). Here, we showed that tumor-specific CTLs execute antitumor cytotoxicity through LTβR and thereby revealed that LTβR, in addition to the perforin and Fas pathways, mediates another cell contact–dependent antitumor effector mechanism.

Recent *in vivo* studies have indicated that T_{reg} cells might play a role in selectively inhibiting the perforin pathway *in vivo*. Chen et al. (37) reported that T_{reg} cells effectively inhibited tumor-specific CTL-mediated tumor rejection whereas exhibited no inhibitory effects on CTL proliferation *in vivo*. Mempel et al. (38) further showed that the failure of the activated tumor-specific CTL to kill tumor cells *in vivo* was correlated with the impaired release of lytic granules. Therefore, selective inhibition of the perforin pathway by immunosuppressive cells *in vivo* might underlie the limited antitumor efficacy of the perforin pathway *in vivo* against certain tumors. If immunosuppressive cells do selectively suppress the perforin pathway, then other cytotoxic pathways, including Fas and the LTβR pathways, could become more critically important in the suppression of tumor development or growth *in vivo*. This may explain the phenomenon of differential antitumor efficacy of

the perforin pathway *in vitro* and *in vivo* and the significant role of perforin-independent cytotoxicity (3).

Immunohistochemical analysis of clinical tumor tissues has shown that 87% to 96% of colorectal, lung, larynx/pharynx, stomach, and melanoma tumors were LTβR-positive, and ~50% of breast tumors showed certain degrees of LTβR staining by anti-LTβR mAb (19). Therefore, it seems that LTβR is expressed in a broad range of solid tumors of diverse tissue origins and histologies. More importantly, the high frequency of LTβR expression is well-correlated with the sensitivity of tumor cells to LTβR-mediated apoptosis. Engagement of LTβR with recombinant ligand protein complex LTα1LTβ2 or LIGHT, or with agonistic anti-LTβR mAb effectively induced apoptotic death of tumor cells *in vitro* (15, 17, 18, 39) and suppressed tumor growth *in vivo* (19, 40). Therefore, LTβR is a common cell surface death receptor of tumor cells. Interestingly, the finding that LTβR functions not only in the homeostasis of immune cells but also in tumor cell apoptosis resembles what is known for Fas, another member of the TNFR superfamily. Like LTβR, Fas was originally identified as a critical factor for homeostasis and self-tolerance of immune cells (41). It has since then been revealed that Fas is widely expressed in various tumors and functions as an essential mediator of extrinsic apoptosis (42). Engagement of Fas with recombinant FasL or agonistic anti-Fas mAb induced apoptosis of tumor cells (43–45). Therefore, although their signaling pathways are distinct, LTβR and Fas are two common death receptors that mediate apoptosis and CTL-induced cytotoxicity in tumor cells.

The cell surface–bound heterotrimeric LTα1LTβ2 complex and the membrane-anchored homotrimeric LIGHT complex are the two ligands that initiate signaling through the LTβR (14). Both ligands are expressed on activated T lymphocytes (28, 33, 46). What we observed here is a coordinate activation of ligands for Fas and LTβR. The activation kinetics of FasL mimics LTα and LIGHT during tumor-specific CTL activation. Moreover, this coordinate ligand activation kinetics was also observed in primary $CD8^+$ T cells, suggesting that coordinate FasL, LTα, and LIGHT activation is a general phenomenon of T cell activation. Because FasL activation is a characteristic of CTL activation, including antitumor immune response (47), the synchronized activation of FasL, LTα, and LIGHT suggest that the LTβR-mediated effector mechanism might be part of an adaptive immune response. Thus, in addition to perforin- and Fas-mediated cytotoxicity, the LTβR-mediated signaling pathway represents another cell contact–dependent cytotoxic mechanism of activated T lymphocytes.

Although both engage LTβR, experimental data obtained from LTα, LTβ, and LTβR-deficient mice indicate that LTα1LTβ2 and LIGHT are not redundant in the development of lymphoid tissues (48–51). It is clear that both LTα1LTβ2 and LIGHT protein complexes are capable of inducing tumor cell apoptosis, but it is not clear whether the functions of LTα1LTβ2 and LIGHT of activated CTL in promoting tumor cell death are distinct under physiologic conditions. Mauri et al. (52) showed that the activation signals required for activation of LIGHT and LTα1LTβ2 by T lymphocytes are different. They observed that LIGHT activation requires stimulation with both phorbol 12-myristate 13-acetate and calcium ionophore, whereas LTα1LTβ2 expression requires only phorbol 12-myristate 13-acetate and thus suggest that these two ligands are important for different T cell functions (52). We observed here that LIGHT and LTα are coordinately activated in tumor-specific T cells by stimulation with tumors, suggesting that these two ligands might both be involved in T cell–directed LTβR-mediated antitumor cytotoxicity *in vivo*.

Although our data showed that TNFα and IFN-γ does not directly induce the apoptosis of CMS4 sarcoma tumor cells *in vitro*, these data do not imply that these cytokines do not function *in vivo* in the induction of tumor cell death. In fact, other TNFR family members have been shown to possess potent antitumor activity (53, 54). However, the relative contribution of LTβR and other TNFR family members in the suppression of tumor development and whether these perforin- and Fas-independent pathways cooperate to inhibit tumor growth require further study.

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