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 adaptively 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+ T cells. 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. Dobrzenski et al. (11) reported that although tumor cytosis 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...
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-2b) mice were obtained from Charles River Laboratories. Female perforin-deficient (ppp) 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 passage in the lungs of normal BALB/c mice, as described (20, 21). The CMS4-metsubline background were kindly provided by M. Smyth (Peter MacCallum River Laboratories. Female perforin-deficient (pfp) mice on a BALB/c background were housed, maintained, and studied in accordance with the approved guidelines of the NIH and Medical College of Georgia for animal use and handling.

Cell surface marker analysis. Tumor cells were immunostained with fluoroconjugated anti-Fas mAb (PharMingen) or an isotopo-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 TNFαR (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).

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

| Primer | Forward | Reverse |
|--------|---------|---------|
| Fas L  | 5'-CTTGGGGCTCTCCAGGGTCACTG-3' | 5'-CTCTCCATAGCAAGGATC-3' |
| Granzyme A | 5'-GCACCACAAATCCAGAAGG-3' | 5'-CAGATCCCCCCCCCAG-3' |
| Granzyme B | 5'-ACCCTTGGCTTGGTACGG-3' | 5'-GTACCAATCTGGTGCG-3' |
| gp70 | 5'-ATGGCTTGTCCGTGTTACTG-3' | 5'-GCTTCTGGATGTCATGCC-3' |
| IFNγ | 5'-TGCCAGCAAGCGTAGCC-3' | 5'-CCGTCACCAGCAGGCAC-3' |
| LTα | 5'-ATGGCAGCTGGCCAGGCTCC-3' | 5'-CCGTCACCAGCAGGCAC-3' |
| LTβ | 5'-TGCCAGCAAGCGTAGCC-3' | 5'-CCGTCACCAGCAGGCAC-3' |
| LIGHT | 5'-GGCCATGAGGAAGACCGTCTG-3' | 5'-GCTTCTGGATGTCATGCC-3' |
| Perforin | 5'-TGCAGAAGGCTATCTCCCTCAG-3' | 5'-CCGTCACCAGCAGGCAC-3' |
| TNFα | 5'-TGCAAGCCTGCTGAGCAGCG-3' | 5'-CCGTCACCAGCAGGCAC-3' |
inhibitor vFLIP. Nontransfected (CMS4-met), vector-transfected (CMS4-met.vector), and vFLIP-transfected (CMS4-met.vFLIP) tumor cells were injected i.v. into naive 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-2Ld-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-2Ld (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 sfSIL (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, naive BALB/c mice were sublethally irradiated (5 Gy) to inactivate host immune cells. CMS4-met.vFLIP cells (2.5 × 10^6 cells/mouse) were injected i.v. into nonirradiated (control) and irradiated mice. Survival was recorded. E, CMS4-met.vFLIP cells (2.5 × 10^6 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.](www.aacrjournals.org Clin Cancer Res 2007;13(17) September 1, 2007 5205)
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 LIGIT 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 LIGIT 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 LIGIT 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 tumor cell surface. Therefore, it is clear that the key molecules involved in these three pathways were coordinately activated during CTL activation.

**Fig. 3.** IFN-γ and TNFα elicited no direct antitumor activity. A, expression of IFN-γ and TNFα in pfp CTLs and IFN-γ and TNFα 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-γ and TNFα 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 and 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. Columns, mean; bars, SD.
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 pfCTLs were then adoptively transferred to the tumor-bearing mice. It is important to point out here that a higher concentration of pfCTL (3 × 10^6/mouse) was used (Fig. 6C) as compared with Figs. 1 and 2 (2.5 × 10^6 and 1 × 10^6 cells/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 pfCTL 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 pfCTLs as compared with CMS4-met.vFLIP.psiRNA.scramble tumor cells (P = 0.001; Fig. 6C). Therefore, we concluded that the pfCTLs 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. 4. Fas-resistant 4T1 mammary carcinoma cells are susceptible to pfCTL-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-2Ld levels by flow cytometry. H-2Ld-specific staining (solid line). Staining with mAb isotype control (shaded area). B, susceptibility of Fas-resistant 4T1.vFLIP tumor cells to pfCTLs 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, pfCTLs (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.](#)

![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, FasL, 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 naive 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 pfCTLs in the presence of neutralizing anti-LTβR mAb. Medium control (medium) and isotype-matched IgG (IgG) were used as controls. Columns, means; bars, SD.](#)
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 Treg cells might play a role in selectively inhibiting the perforin pathway in vivo. Chen et al. (37) reported that Treg 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 LT\(\beta\)R-positive, and ~50% of breast tumors showed certain degrees of LT\(\beta\)R staining by anti-LT\(\beta\)R mAb (19). Therefore, it seems that LT\(\beta\)R is expressed in a broad range of solid tumors of diverse tissue origins and histologies. More importantly, the high frequency of LT\(\beta\)R expression is well-correlated with the sensitivity of tumor cells to LT\(\beta\)R-mediated apoptosis. Engagement of LT\(\beta\)R with recombinant ligand protein complex LT\(\alpha\)LT\(\beta\)2 or LIGHT, or with agonistic anti-LT\(\beta\)R mAb effectively induced apoptotic death of tumor cells in vitro (15, 17, 18, 39) and suppressed tumor growth in vivo (19, 40). Therefore, LT\(\beta\)R is a common cell surface death receptor of tumor cells. Interestingly, the finding that LT\(\beta\)R 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 LT\(\beta\)R, 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, LT\(\beta\)R and Fas are two common death receptors that mediate apoptosis and CTL-induced cytotoxicity in tumor cells.

The cell surface–bound heterotrimERIC LT\(\alpha\)LT\(\beta\)2 complex and the membrane-anchored homotrimERIC LIGHT complex are the two ligands that initiate signaling through the LT\(\beta\)R (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 LT\(\beta\)R. The activation kinetics of FasL mimics LT\(\alpha\) 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, LT\(\alpha\), 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, LT\(\alpha\), and LIGHT suggest that the LT\(\beta\)R-mediated effector mechanism might be part of an adaptive immune response. Thus, in addition to perforin- and Fas-mediated cytotoxicity, the LT\(\beta\)R-mediated signaling pathway represents another cell contact–dependent cytotoxic mechanism of activated T lymphocytes.

Although both engage LT\(\beta\)R, experimental data obtained from LT\(\alpha\), LT\(\beta\), and LT\(\beta\)-deficient mice indicate that LT\(\alpha\)LT\(\beta\)2 and LIGHT are not redundant in the development of lymphoid tissues (48–51). It is clear that both LT\(\alpha\)LT\(\beta\)2 and LIGHT protein complexes are capable of inducing tumor cell apoptosis, but it is not clear whether the functions of LT\(\alpha\)LT\(\beta\)2 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 LT\(\alpha\)LT\(\beta\)2 by T lymphocytes are different. They observed that LIGHT activation requires stimulation with both phorbol 12-myristate 13-acetate and calcium ionophore, whereas LT\(\alpha\)LT\(\beta\)2 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 LT\(\alpha\) 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 LT\(\beta\)R-mediated antitumor cytotoxicity in vivo.

Although our data showed that TNF\(\alpha\) and IFN-\(\gamma\) 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 LT\(\beta\)R 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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### References

1. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoeediting. Annu Rev Immunol 2004;22:329–60.

2. Medema JP, de Jong J, van Hall T, Melief CJ, Offringa R. Escape of tumors in vivo by expression of cellular FLICE-inhibitory protein. J Exp Med 1999;190:1033–8.

3. Seki N, Brooks AD, Kagi D, CR et al. 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. J Immunol 2002;168:3484–92.

4. Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DR, Trapani JA. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. J Exp Med 2000;192:755–60.

5. van den Broek ME, Kagi D, Ossendorf F, et al. Decreased tumor surveillance in perforin-deficient mice. J Exp Med 1996;184:1781–90.

6. Caldwell SA, Ryan MH, McDuffie E, Abrams SI. The Fas/Fas ligand pathway is important for optimal tumor immune response in a mouse model of CTL adoptive immunotherapy of experimental CMS4 lung metastases. J Immunol 2003;171:2402–12.

7. Kagi D, Liedemann B, Burki K, et al. Cytotoxic mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature 1994;369:31–7.

8. Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. Nat Rev Immunol 2006;6:940–52.

9. Pohelchin CH, Hu HM, Yamada J, et al. TNF plays an essential role in tumor regression after adoptive transfer of perforin/FasL double knockout effector T cells. J Immunol 2003;170:2004–13.

10. Baba A, Chatterjee SK, Foon KA, Bhattacharya-Dobrzanski MJ, Reiome JB, Hollenaugh JA, Hyland JC, Dutton RW. Effector cell-derived lymphokine a and Fas ligand, but not perforin, promote Tc1 and Tc2 effector cell-mediated tumor therapy in established pulmonary metastases. Cancer Res 2004;64:408–14.

11. Winter H, Hu HM, Urba WJ, Fox BA. Tumor regression after adoptive transfer of effector T cells is independent of perforin or Fas ligand (APO-TC/CD95L). J Immunol 1999;163:4462–72.

12. Lee SH, Bar-Haim E, Machlenkin A, et al. In vivo rejection of tumor cells dependent on CD8 cells that kill independently of perforin and FasL. Cancer Gene Ther 2004;11:237–48.

13. Ware CF. Network communications: lymphotixin, LIGHT, and TNF. Annu Rev Immunol 2005;23:787–819.

14. Browning JL, Miatkowski K, Sizing I, et al. Signaling through the lymphotixin-\(\beta\) receptor induces the death of some adenocarcinoma tumor lines. J Exp Med 1996;183:867–78.

15. Chen MC, Hwang MJ, Chou YC, et al. The role of apoptosis signal-regulating kinase 1 in lymphotixin-\(\beta\) receptor-mediated cell death. J Biol Chem 2003;278:16073–81.

16. Rooney JA, Butovich KD, Glass AA, et al. The lymphotixin-\(\beta\) receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. J Biol Chem 2000;275:14307–16.
27. Ashkenazi A. Targeting death and decoy receptors. Cancer Res 2005;65:4376–88.

28. Sinha P, Clements VK, Ostrand-Rosenberg S. Inter-Yamaguchi T, Sakaguchi S. Regulatory T cells in immune selection and emergence of aggressive tumor variants as a consequence of Fas-mediated cytotoxicity and altered IFN-γ-regulated gene expression. Cancer Res 2005;65:4376–88.

29. Liu K, Caldwell SA, Abrams SI. Immune selection and elimination of IFN-γ regulatory factor 8 by DNA methylation is a molecular determinant of apoptotic resistance and metastatic phenotype in metastatic tumor cells. Cancer Res 2007;67:3301–9.

30. Liu K, Caldwell SA, Abrams SI. CTL Adaptive immunotherapy concurrently mediates tumor regression and tumor escape. J Immunol 2006;176:3374–82.

31. Futterer A, Mink K, Luz A, Kosco-Vilbois MH, Futterer A, Mink K, Luz A, Kosco-Vilbois MH, et al. Targeting the lymphotoxin-α receptor with agonist antibodies as a therapeutic strategy for一秒秒ectomy and lymphotoxin-β receptor signaling complex: role of the tumor necrosis factor superfamily that induces apoptosis. Immunity 1995;3:673–82.

32. Strasser A. O’Connor L. Dixit VM. Apoptosis signaling. Annu Rev Biochem 2000;69:217–45.

33. Pesk SW. Promoting apoptosis as a strategy for cancer drug discovery. Nat Rev Cancer 2005;5:876–85.

34. Park HH, Lo YC, Lin SC, Wang L, Yang JK, Wu H. The death domain superfamily in intracellular signaling of apoptosis and inflammation. Annu Rev Immunol 2007;25:561–86.

35. Ware CF, vanArsdale TL, Crowe PD, Browning JL. The ligands and receptors of the lymphotoxin system. Curr Top Microbiol Immunol 1995;198:175–218.

36. Rennert PD, James D, Mackay F, Browning JL, Hochman PS. Lymph node genesis is induced by signaling through the lymphotoxin-γ receptor. Immunity 1998;9:71–9.

37. Futterer A, Mink K, Luz A, Kosco-Vilbois MH, Pfeffer K. The lymphotoxin-γ receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. Immunity 1998;9:59–70.

38. Fu YX, Chaplin DD. Development and maturation of secondary lymphoid tissues. Annu Rev Immunol 1999;17:399–433.

39. Chen ML, Pittet MJ, Gorelik L, et al. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-b signals in vivo. Proc Natl Acad Sci U S A 2005;102:419–24.

40. Mempel TR, Pittet MJ, Khazaie K, et al. Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation. Immunity 2006;25:129–41.

41. Wu MY, Wang PY, Han SH, Hsieh SL. The cytoplasmic domain of the lymphotoxin-γ receptor mediates cell death in HeLa cells. J Biol Chem 1999;274:11868–73.

42. Zerafa N, Westwood JA, Cretney E, et al. Cutting edge: TRAIL deficiency accelerates hematological malignancies. J Immunol 2005;175:5586–90.