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Tumor-Infiltrating CD4$^+$ T Lymphocytes Express APO2 Ligand (APO2L)/TRAIL upon Specific Stimulation with Autologous Lung Carcinoma Cells: Role of IFN-α on APO2L/TRAIL Expression and -Mediated Cytotoxicity[1]

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In the present report, we have investigated TRAIL/APO2 ligand (APO2L) expression, regulation, and function in human lung carcinoma tumor-infiltrating lymphocytes. Using a panel of non-small cell lung carcinoma cell lines, we first showed that most of them expressed TRAIL-R1/DR4, TRAIL-R2/DR5, but not TRAIL-R3/DcR1 and TRAIL-R4/DcR2, and were susceptible to APO2L/TRAIL-induced cell death. Two APO2L/TRAIL-sensitive tumor cell lines (MHC class I$^+$/II$^+$ or I$^+$/II$^-$) were selected and specific CD4$^+$ HLA-DR- or CD8$^+$ HLA-A2-restricted CTL clones were respectively isolated from autologous tumor-infiltrating lymphocytes. Interestingly, although the established T cell clones did not constitutively express detectable levels of APO2L/TRAIL, engagement of their TCR via activation with specific tumor cells selectively induced profound APO2L/TRAIL expression on the CD4$^+$, but not on the CD8$^+$, CTL clones. Furthermore, as opposed to the CD8$^+$ CTL clone which mainly used granule exocytosis pathway, the CD4$^+$ CTL clone lysed the specific target via both perforin/granzymes and APO2L/TRAIL-mediated mechanisms. The latter cytotoxicity correlated with APO2L/TRAIL expression and was significantly enhanced in the presence of IFN-α. More interestingly, in vivo studies performed in SCID/nonobese diabetic mice transplanted with autologous tumor and transferred with the specific CD4$^+$ CTL clone in combination with IFN-α resulted in an important APO2L/TRAIL-mediated tumor growth inhibition, which was prohibited by soluble TRAIL-R2. Our findings suggest that APO2L/TRAIL, specifically induced by autologous tumor and up-regulated by IFN-α, may be a key mediator of tumor-specific CD4$^+$ CTL-mediated cell death and point to a potent role of this T cell subset in tumor growth control. ***The Journal of Immunology*****, 2002, 169: 809–817.**

Abnormal regulation of apoptosis has been described to be implicated in tumor development and resistance to cancer therapies. Therefore, understanding the cellular mediators of apoptosis implicated in tumor growth control may lead to the development of new cancer therapeutic approaches. TRAIL/APO2 ligand (APO2L)[3] is a recently identified type II integral membrane protein belonging to the TNF family (1, 2). Like TNF and Fas ligand (FasL), APO2L/TRAIL plays an important role in regulating immune response and has been shown to be a potent inducer of apoptosis in various tumor cell lines (1, 2). As opposed to administration of TNF and FasL, which are both toxic and lethal for mice (3, 4), APO2L/TRAIL exerts potent in vivo antitumor activity in mice and nonhuman primates without any systemic toxicity (5, 6).

APO2L/TRAIL can induce apoptosis in a wide range of transformed cell lines but not in normal cells (1, 2, 5, 6) following interaction with TRAIL receptor (TRAIL-R) 1/DR4 or TRAIL-R2/DR5 death domain (DD)-containing receptors (7–11). However, it has been described that it may participate in normal activation-induced cell death processes of T cells (12) and may regulate peripheral tolerance and prevention of autoimmunity (13). Furthermore, it has been recently reported that APO2L/TRAIL may induce apoptosis of normal human hepatocytes (14), although this last observation seemed to depend on the preparation of soluble APO2L/TRAIL used (15). DR4 and DR5 receptors are type I transmembrane proteins believed to be negatively regulated by the binding of APO2L/TRAIL to two additional receptors, TRAIL-R3/DcR1/TRID (8, 9, 16) and TRAIL-R4/DcR2/TRUNDD (17–19). TRAIL-R3, an extracellular GPI-linked protein without an intracellular domain and TRAIL-R4, a type I transmembrane protein with a truncated cytoplasmic DD, have been described to inhibit apoptosis by acting as decoy receptors (8, 9, 17–19). It has been proposed that APO2L/TRAIL preferentially induces apoptosis in transformed cells but not in normal cells, possibly due to the selective expression of TRAIL-R3 and/or TRAIL-R4 in the latter cells (20). However, more recent data indicated that APO2L/TRAIL decoy receptors are not the predominant mechanisms to explain the resistance of normal cells to APO2L/TRAIL cytotoxicity

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[3] Abbreviations used in this paper: APO2L, APO2 ligand; FasL, Fas ligand; TRAIL-R, TRAIL receptor; DD, death domain; TIL, tumor-infiltrating lymphocyte; NOD, nonobese diabetic; ADC, adenocarcinoma; LCC, large cell carcinoma; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma; sTRAIL, soluble APO2L/TRAIL; rh, recombinant human.

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(20). Indeed, it has been reported that cellular susceptibility to DR4/DR5-induced apoptosis is regulated at the intracellular level rather than at the receptor level mainly by cellular FLIP expression (20, 21).

CTL play an important role in immune surveillance against tumors. It has been generally admitted that CTL kill target cells via two distinct effector pathways. The major one is a secretory mechanism involving receptor-triggered exocytosis of perforin and granzyme proteins. The second is based on TCR-induced surface expression of FasL on effector cells, which cross-links the Fas receptor on target cells and induces apoptosis via DD-mediated recruitment of caspases (3, 22–24). More recently, it has been reported that APO2L/TRAIL might constitute an additional pathway of T cell-mediated cytotoxicity against some tumor cells including melanoma and Jurkat T cell lymphoma (25, 26). Indeed, APO2L/TRAIL has been described to induce apoptosis in FasL-resistant melanoma cells by CD4$^+$ CTL (25). Furthermore, it has been described to be involved in the surveillance of tumor development and metastasis by NK cells (27–30). In addition, evidence has been provided indicating that IFN-α may enhance APO2L/TRAIL expression and nonspecific cytotoxic activity of anti-CD3-stimulated peripheral blood T lymphocytes toward allogeneic renal cell carcinoma (31). In the present study, we asked whether APO2L/TRAIL could be expressed by human lung carcinoma tumor-infiltrating lymphocytes (TIL) and whether it could be used by TIL-derived CD4$^+$ and CD8$^+$ CTL clones to exert TCR-dependent MHC-restricted T cell-mediated cytotoxic activity toward autologous tumor cells. Our data demonstrate that upon TCR recognition, specific tumor cells strongly induced APO2L/TRAIL expression on CD4$^+$, but not on CD8$^+$, CTL clones. This expression was slightly increased in the presence of IFN-α. Interestingly, APO2L/TRAIL-dependent cytotoxicity mediated by the CD4$^+$ CTL clone toward its autologous tumor was significantly up-regulated by IFN-α in vitro as well as in vivo in a SCID/nonobese diabetic (NOD) mice model, leading to tumor growth inhibition.

Materials and Methods

Patient characteristics and clinical course

Patient Pub, a 65-year-old Caucasian man, presented in 1996 with a differentiated adenocarcinoma (ADC) of the left lung. No other tumoral localizations were found and the primary tumor (pT2, N0) was resected. The patient received no other treatment before the surgery and deceased in 2000.

Patient Bla, a 57-year-old Caucasian woman, presented in 1996 with a large cell carcinoma (LCC) of the right lung (pT2, N0) and an ADC of the left lung (pT2, N1). The primary tumor of the right lung was resected. The patient received no other treatment before the surgery and deceased in 1998.

Derivation and culture of CTL clones

Non-small cell lung cancer (NSCLC) cell lines Institut Gustave Roussy (IGR)-Pub and IGR-B2 were derived from the biopsies of patient Pub ADC (HLA-A2, B7/Bw6, Cw7, DRB1/DRB15, DRw51, DQ6) and patient Bla LCC (A2/68, B35/38, Cw4/Cw12), respectively, as previously described (32). IGR-Pub and IGR-B2 cells were maintained in DMEM/F12 medium supplemented with 10% FCS (Seromed, Berlin, Germany), 1% Ultroser G (Life Technologies, Cergy Pontoise, France), and 1% penicillin-streptomycin.

CTL clones, P62 and B90, were derived as previously described (33). Briefly, fresh tumor samples were dissociated in DMEM containing 1 mM HEPES, 0.3 U/ml DNase, 0.5 U/ml collagenase, and 0.28 U/ml hyaluronidase (Life Technologies) and the resulting cell suspension was frozen. After thawing, viable TIL were seeded at 10^4 cells/microwell and stimulated by the addition of irradiated (10,000 rad) autologous tumor cells (3×10^3/well) and irradiated allogeneic Laz509 EBV-transformed B cells (4×10^4/well) in RPMI 1640 medium supplemented with 10% human AB serum (Institut Jacques Boy, Reims, France) and rIL-2 (20 U/ml; Roussel-Uclaf, Romainville, France). Cells were fed every 3 days with medium and IL-2, and restimulated every other week with irradiated autologous tumor cells and irradiated allogeneic EBV-B cells. After 4 wk, the resulting cell lines were cloned by limiting dilution (0.5 cell/well) in 96-well V-shaped microtiter plates (Nunc, Roskilde, Denmark), in the presence of irradiated autologous tumor and Laz509 cells, rIL-2 (100 U/ml), and 3% of conditioned medium from PHA-activated lymphocytes. CTL clones P62 and B90 were restimulated every other week with the same protocol.

Allogeneic NSCLC tumor cell lines

The IGR-Heu tumor cell line was established from a Heu patient suffering from LCC of the lung as described previously (32). The LCC-M4 cell line was established in the laboratory in 1999 from LCC. A549 (ADC), SK-MES, and Ludlu (squamous cell carcinoma (SCC)) cell lines were purchased from the European Collection of Cell Cultures (Salisbury, U.K.). H1155 (LCC), H1355 (ADC), H820 and H460 (LCC) were kindly provided by S. Rogers (Brigham and Women's Hospital, Boston, MA) (34). All tumor cell lines were cultured in DMEM/F12 medium containing 10% heat-inactivated FCS, 1% Ultroser G (Life Technologies) and 1% penicillin-streptomycin, at 37°C in a humidified atmosphere with 5% CO2.

Abs and reagents

The mAbs directed against APO2L/TRAIL (M181) and TRAIL receptors, TRAIL-R1 (M271), TRAIL-R2 (M413), TRAIL-R3 (M430), and TRAIL-R4 (M445) were kindly provided by Immunex (Seattle, WA). Anti-CD95/Fas antagonistic mAb (ZB4), FITC-conjugated goat anti-mouse IgG Abs, biotin-conjugated goat anti-mouse IgG Abs, PE-conjugated streptavidin and the mouse isotypic control IgG Abs were purchased from Immunotech (Marseille, France). Recombinant human soluble APO2L/TRAIL (sTRAIL) was provided by Genentech (San Francisco, CA) and recombinant human (rh) TRAILR2-Fc (TR2-Fc) by Immunex. rhIg-Fc irrelevant Fc fusion protein (CD33 signal peptide-Fc molecule) was purchased from R&D Systems (Abingdon, U.K.).

Tumor cell sensitivity to APO2L/TRAIL-induced cell death

The sensitivity of lung tumor cell lines to APO2L/TRAIL-mediated cell death was measured by a conventional ^{51}Cr release assay using triplicate cultures in round-bottom 96-well plates. Three $\times$ 10^3 chromium-labeled target cells (100 μl) were seeded per well and sTRAIL (100 μl) was added to a final concentration of 10, 50, 200, or 500 ng/ml. After a 14-h incubation at 37°C, 100 μl of supernatant were harvested and counted in a gamma counter. Percent of lysis was calculated conventionally.

Flow cytometry analysis

Cells (3×10^5) were incubated with various anti-TRAIL-R mAbs (3 μg/ml) or isotypic control for 30 min at 4°C, followed by FITC-conjugated goat anti-mouse Abs. After washing with PBS, cells were fixed with 1% formaldehyde and analyzed on a FACSCalibur flow cytometer and data were processed using CellQuest software (BD Biosciences, San Jose, CA). For APO2L/TRAIL expression analysis, T cells were left untreated or cocultured (E:T ratio, 1:1) with autologous or allogeneic tumor cells for the indicated time period in the presence or absence of IFN-α (200 U/ml). Cells were then incubated with M181, anti-APO2L/TRAIL mAb (4 μg/ml), or isotypic control Ab, followed by biotinylated goat anti-mouse IgG and then PE-labeled streptavidin. In coculture experiments, a staining with anti-human CD3 mAb (OKT3) was included to identify CTL.

Cytotoxicity assays

The cytotoxic activity of P62 or B90 CTL clones was measured by a conventional 6-h ^{51}Cr release assay using triplicate cultures in round-bottom 96-well plates. E:T ratios were as indicated, on 3000 target cells/well. Percent specific cytotoxicity was calculated conventionally. Inhibition of lysis by anti-MHC class I (W6/32), anti-HLA-A2.1 (MA2.1), anti-MHC-class II (9-49), and anti-HLA-DR (L24.3) mAbs was tested by preincubating target cells for 2 h with saturating concentrations of Ab or ascitic fluid. Blockade of APO2L/TRAIL pathway was performed by preincubating target cells for 2 h with anti-TRAIL-R1 (M271) + anti-TRAIL-R2 (M413) mAbs (10 μg/ml each), or by adding rhTRAILR2-Fc (10 μg/ml) during the assay. The IFN-α effect on cytotoxic activity of TIL clones was measured either by preincubation of T cells with IFN-α (200 U/ml) followed or not by washing before their addition to the assay, or by its addition during the assay without preincubation. EGTA (4 mM) and MgCl2 (2 mM) added during the assay were used to inhibit Ca^{2+}-dependent perforin/granzyme-mediated lysis. Anti-Fas-neutralizing ZB4 mAb was used in similar conditions than anti-TRAIL-R mAbs to block CD95 pathway, as previously described (35).

In vivo CTL clone transfer experiments

Six- to 8-wk-old SCID/NOD mice were engrafted s.c. into the right flank with tumor biopsies (36) previously established in vivo in nude mice by

Table I. *Flow cytometry analysis of TRAIL-R expression on NSCLC cell lines*[a]

Cell Line	Histological Type	TRAIL-R1 (%)	TRAIL-R2 (%)	TRAIL-R3 (%)	TRAIL-R4 (%)
IGR-Heu	LCC	3	1	1	0
IGR-B1	LCC	56 (9)	20 (6)	8 (5)	1
IGR-B2	LCC	75 (13)	52 (9)	12 (6)	1
H460	LCC	73 (9)	90 (13)	0	0
H1155	LCC	95 (21)	59 (8)	1	1
Ludlu	SCC	86 (11)	18 (5)	0	0
SKMES	SCC	62 (9)	63 (9)	0	1
IGR-Pub	ADC	79 (9)	75 (8)	1	1
H1355	ADC	74 (10)	17 (5)	1	1
A549	ADC	56 (10)	60 (10)	2	0

[a] Staining with anti-TRAIL-R mAbs was performed as described in *Materials and Methods.* Percentages of positive cells are indicated. Numbers in parentheses correspond to mean fluorescence intensity of specific staining.

injection of IGR-Pub cells. After 7 days, mice bearing tumors of ~50 mm^3 were injected intratumorally with autologous P62 $CD4^+$ CTL clone (5×10^6 cells/mouse) or with control medium, and the same injection was repeated 10 days later (J17). IFN-α (5,000 or 25,000 U/mouse/injection), IFN-α + rhTRAILR2-Fc (25,000 U + 70 μg/mouse/injection) or control medium was then injected in mice every 2–3 days from days 8–27. Five mice per group were monitored for tumor growth, and tumor volume was calculated according to the following formula: volume = 0.5 × longest diameter × (shortest diameter)^2.

Results

NSCLC tumor cell lines display heterogeneous APO2L/TRAIL-R expression and sensitivity

Initial experiments were performed to investigate TRAIL-R expression on a panel of human NSCLC tumor cell lines including 5 LCC, 2 SCC, and 4 ADC cell lines. Immunofluorescence analysis indicated that all tumor cell lines expressed TRAIL-R1 (DR4) and TRAIL-R2 (DR5) molecules except IGR-Heu, which only expressed very low intensity of TRAIL-R1, and IGR-B1, Ludlu, and H1355, which weakly expressed TRAIL-R2 (Table I). In contrast, all tumor cell lines failed to express TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) except IGR-B1 and IGR-B2, which were slightly positive for TRAIL-R3 (Table I).

We then investigated the susceptibility of NSCLC cell lines to APO2L/TRAIL-mediated apoptosis using sTRAIL at concentrations ranging from 10 to 500 ng/ml, as previously reported (5, 6, 25). Data depicted in Fig. 1 indicate that the majority of NSCLC cell lines were sensitive to APO2L/TRAIL-induced apoptosis. This sensitivity varied from one cell line to another with a maximal tumor cell lysis obtained at 200 or 500 ng/ml. In contrast, the IGR-Heu cell line, deficient for DR4 and DR5 expression, was completely resistant to APO2L/TRAIL-mediated cell death. Among the sensitive tumor cell lines two, IGR-Pub and IGR-B2, were selected for further studies.

Isolation and characterization of tumor-reactive $CD4^+$ and $CD8^+$ CTL clones

The NSCLC cell lines, IGR-Pub (MHC class I^+/II^+) and IGR-B2 (MHC class I^+/II^-), were derived from primary tumor biopsies. Mononuclear cells that infiltrated the tumors were isolated and stimulated with irradiated autologous tumor cell line, irradiated allogeneic EBV-transformed B cells, and IL-2. On day 28, the responder lymphocytes of each TIL suspension were cloned by limiting dilution and several tumor-specific CTL clones were obtained. Two isolated CTL clones, P62 and B90 ($CD3^+$, TCR$\alpha\beta^+$), expressed respectively a $CD4^+$ or a $CD8^+$ phenotype. Both T cell clones were $CD28^-$, $CCR7^-$, $CD45RA^-$, and $CD45RO^+$ indicating that they belong to effector memory T cells (data not shown).

P62 and B90 CTL clones specifically lysed the autologous tumor cell line but not the autologous EBV-B cells or PHA-blasts, nor the allogeneic NSCLC cell lines and the K562 NK-target cells (Fig. 2*A* and data not shown). The cytotoxic activity of P62 CTL clone toward IGR-Pub autologous tumor cell line was inhibited by anti-class II (9–49) and anti-HLA-DR (L24.3) mAbs indicating that it recognizes its specific target in a HLA-DR-restricted manner (Fig. 2*B* and data not shown). The cytotoxicity of B90 clone toward IGR-B2 autologous tumor cells was inhibited by anti-class I (W6/32) and anti-HLA-A2.1 (MA2.1) mAb, indicating that it is HLA-A2.1-restricted (Fig. 2*B* and data not shown). The lytic activity of both T cell clones toward their respective target cells was blocked in the presence of anti-TCR$\alpha\beta$ and anti-CD3 mAb, supporting that it is TCR-mediated (data not shown).

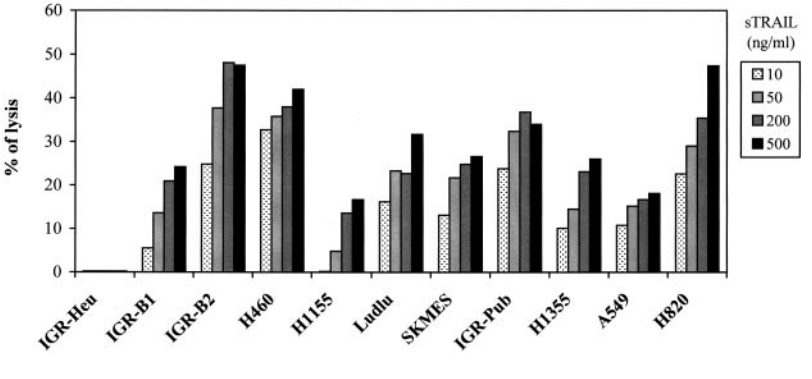

FIGURE 1. Sensitivity of NSCLC cell lines to APO2L/TRAIL-mediated cytotoxicity. Cells were incubated for 14 h in the presence of indicated concentrations of sTRAIL. Percentage of lysis was determined using a ^{51}Cr release assay performed in triplicate.

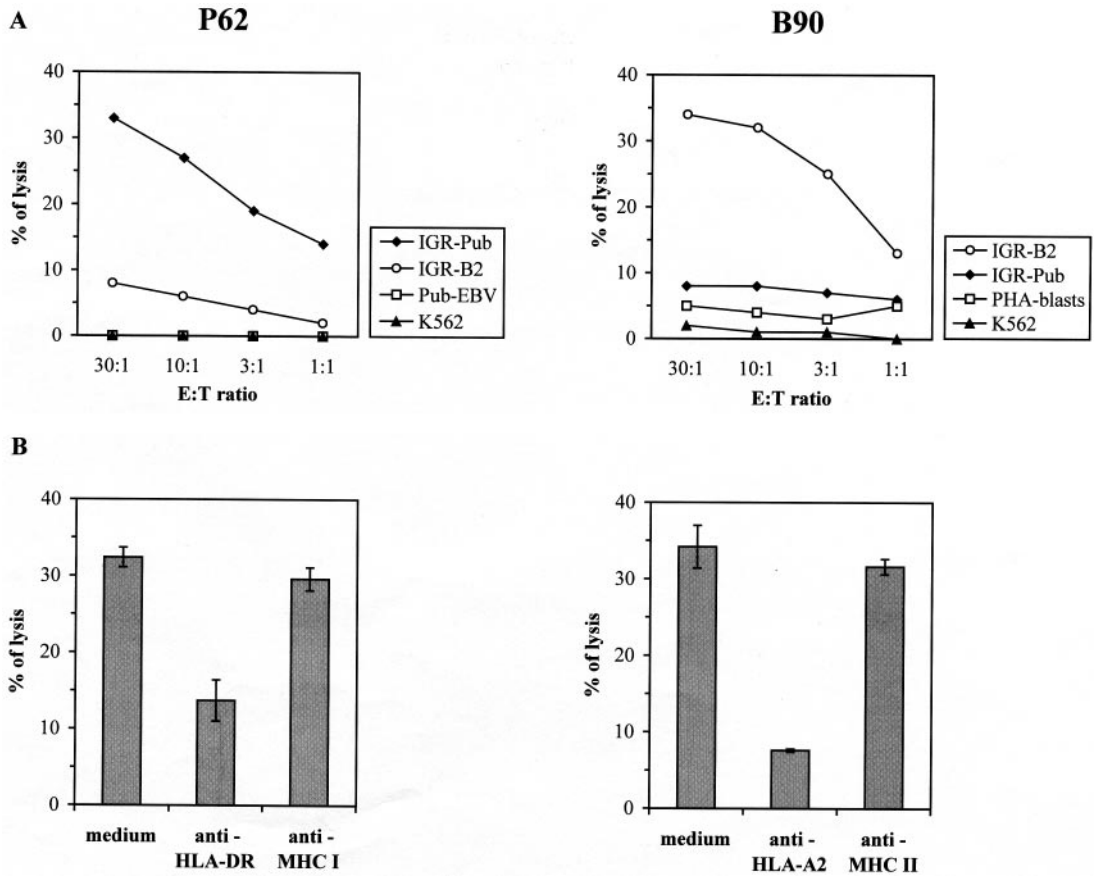

FIGURE 2. *A*, Lytic activity of P62 CD4+ or B90 CD8+ CTL clones toward autologous or allogeneic lung tumor cell lines, autologous EBV-transformed B cells or autologous PHA-blasts, and K562. E:T ratios were as indicated. *B*, Cytolytic activity of P62 and B90 T cell clones toward, respectively, IGR-Pub and IGR-B2 autologous targets. Cytotoxic experiments were performed either in medium or in the presence of anti-MHC class I (W6/32), anti-MHC class II (9-49), anti-HLA-DR (L24.3), or anti-HLA-A2.1 (MA2.1) mAb. ^{51}Cr-labeled tumor cells were preincubated for 2 h with saturating concentrations of anti-HLA mAb, and CTL clones were then added at a 10:1 E:T ratio.

Autologous tumor specific induction of APO2L/TRAIL expression on the CD4+ CTL clone

Cytometry analysis performed with the use of anti-APO2L/TRAIL (M181) mAb showed that P62 and B90 T cell clones did not express APO2L/TRAIL at the basal level (Fig. 3, t_0). Interestingly, after specific stimulation of P62 with IGR-Pub tumor, a profound induction of APO2L/TRAIL protein expression was detected after a 4-h incubation reaching a peak after a 16-h sensitization (Fig. 3*A* and data not shown). A slight but significant and sustained enhancement of APO2L/TRAIL expression was observed after addition of IFN-α during coculture of P62 CD4+ CTL clone with autologous tumor cell line. In contrast, incubation of P62 with IFN-α alone induced only negligible and transient APO2L/TRAIL expression (Fig. 3*A*). Furthermore, T cell clone cocultured with an allogeneic LCC-M4 cell line (MHC class I+/II+) failed to induce APO2L/TRAIL expression and only a transient staining was observed after addition of IFN-α (Fig. 3*B*). To investigate whether CD4+ T cells might harbor APO2L/TRAIL within, and, when stimulated through TCR, express these molecules on the surface, RT-PCR and Western blot experiments were performed on the P62 CTL clone before and after stimulation with the autologous tumor cell line. Our results clearly show that the CD4+ T cells do not significantly express APO2L/TRAIL mRNA and protein before stimulation with autologous tumor, whereas this expression was induced after incubation of the CTL clone with tumor cells (data not shown). These data clearly indicate that APO2L/TRAIL was induced following stimulation through TCR and was not preformed.

With respect to B90 CD8+ CTL, APO2L/TRAIL was undetectable following stimulation with the IGR-B2 autologous tumor cell line. In addition, only a slight and brief APO2L/TRAIL expression was induced after a 2-h incubation of the T cell clone with IFN-α in the presence or absence of the specific tumor cells and felt down after 6 h (Fig. 3*C*). Similar results were obtained with specific CD8+ CTL clones isolated from either Bla or Pub TIL (data not shown).

Optimization effect of IFN-α on tumor-specific CD4+ CTL clone cytotoxic activity

Additional experiments were performed to investigate the functional effect of IFN-α on P62 and B90 CTL clone killing of autologous tumor cells. For this purpose, CTL clone cytotoxic activity toward the specific target was measured in the absence or presence of IFN-α (200 U/ml). Fig. 4*A* shows a significant increase of P62 CTL clone lysis against the IGR-Pub autologous tumor cell line in the presence of IFN-α. This enhancement persists when T cells were preincubated with IFN-α followed by subsequent washing before their addition to the cytotoxicity assay indicating that the effect of IFN-α is exerted on effector cells (Fig. 4*A*). In contrast, preincubation of P62 with IFN-α had a marginal effect on its cytotoxic activity toward the allogeneic APO2L/TRAIL-sensitive LCC-M4 cell line, which was not recognized by the CTL clone (data not shown). Regarding B90 CD8+ CTL, even though the specific IGR-B2 tumor cell line was more sensitive to APO2L/

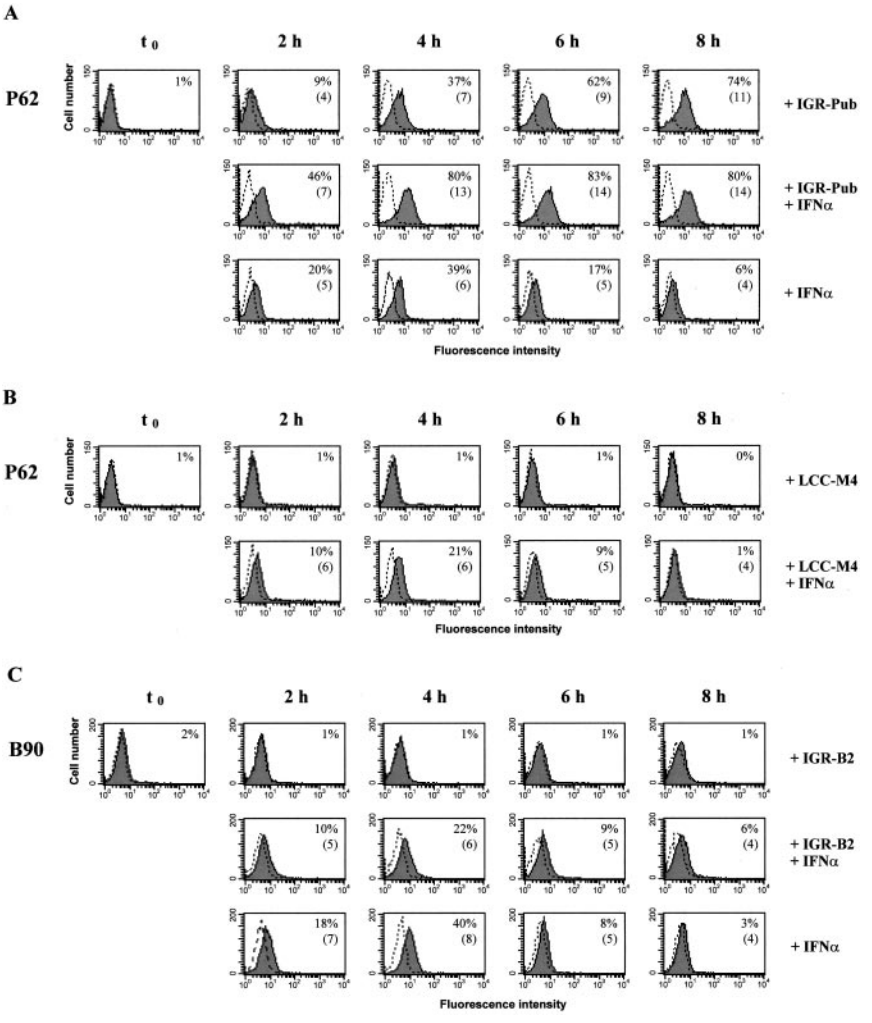

FIGURE 3. Analysis of APO2L/TRAIL expression regulation on P62 $CD4^+$ and B90 $CD8^+$ CTL clones. *A,* Surface APO2L/TRAIL protein expression on the P62 $CD4^+$ CTL clone as determined by immunofluorescence analysis using M181 anti-APO2L/TRAIL mAb (gray). Isotypic control mAb was included (white). T cells were left untreated (t_0), or incubated for indicated time points either with IFN-α alone (200 U/ml) or with IGR-Pub autologous tumor cells at a 1:1 E:T ratio in the absence or presence of IFN-α (200 U/ml). Percentages of positive cells are indicated. Numbers in parentheses correspond to mean fluorescence intensity. *B,* Analysis of APO2L/TRAIL expression on the P62 CTL clone following incubation with allogeneic tumor cell line. Cells were either left untreated (t_0) or incubated for indicated time points with allogeneic (LCC-M4) tumor cells at 1:1 E:T ratio in the absence or presence of IFN-α (200 U/ml). *C,* Surface APO2L/TRAIL protein expression on B90 $CD8^+$ CTL clones. Cells were left untreated (t_0), or incubated for indicated time points either with IFN-α alone (200 U/ml) or with autologous (IGR-B2) tumor cells at a 1:1 E:T ratio in the absence or presence of IFN-α (200 U/ml). Percentages of positive cells and mean fluorescence intensity are indicated as in *A* and *B.*

TRAIL-mediated lysis than IGR-Pub (Fig. 1), only a marginal increase of T cell clone-mediated lysis was observed when IFN-α was added during the cytotoxic assay (Fig. 4*B*). This cytotoxicity was unaffected by the rhTRAILR2-Fc molecule, supporting the lack of APO2L/TRAIL-pathway involvement (data not shown).

To determine whether IFNα-induced increase of P62 cytotoxicity was APO2L/TRAIL-mediated, experiments were performed following preincubation of IGR-Pub target cells with anti-TRAIL-R1 plus anti-TRAIL-R2 mAbs. Initial study was performed to investigate the cytotoxicity pathways used by the P62 CTL clone to kill its specific target. Fig. 5*A* shows that while P62-mediated cytotoxicity was unaffected by ZB4 anti-Fas neutralizing mAb, it was partially inhibited by anti-TRAIL-R mAbs and incompletely blocked in the presence of EGTA (4 mM) and MgCl2 (2 mM), known to block the Ca^{2+}-dependent perforin/granzyme-mediated pathway. Furthermore, Fig. 5*B* clearly shows that IFN-α-induced increase of P62 specific lysis was inhibited in the presence of anti-TRAIL-R mAbs. Concordant results were obtained in effector cell inhibition cytotoxicity experiments performed in the presence of rhTRAILR2-Fc (Fig. 5*C*). In contrast, rhIg-Fc irrelevant Fc-fusion protein used as a negative control has a marginal effect on IFN-α-stimulated P62-mediated lysis (Fig. 5*C*). These results strongly suggest that IFN-α-induced increase of the specific $CD4^+$ T cell cytotoxicity is mainly APO2L/TRAIL-mediated.

In vivo effect of IFN-α on $CD4^+$ CTL clone antitumor activity

The unique feature of IFN-α to up-regulate APO2L/TRAIL-mediated lysis of the $CD4^+$ CTL clone against its autologous tumor prompted us to examine its effect in vivo. For this purpose, the IGR-Pub tumor was engrafted into SCID/NOD mice and P62 CTL were intratumorally transferred 7 days later. Initial experiments indicated that transfer of P62 CTL, previously activated in vitro with autologous tumor and stimulated with IL-2 before injection, significantly inhibited tumor growth in vivo (data not shown). The effect of IFN-α local administration on antitumor activity of transferred P62 CTL was then investigated in the absence of previous stimulation of the T cell clone. Therefore, experiments were performed with effector T cells fed 3 wk earlier with irradiated autologous tumors and starved from IL-2 for 3 days before intratumoral injection. In these particular culture conditions, $CD4^+$ T cells did not express APO2L/TRAIL on their surface (Fig. 3*A*, t_0). Transfer of such nonstimulated P62 $CD4^+$ T cells was unable to induce tumor growth suppression (Fig. 6). In contrast, when specific CTL were transferred in combination with IFN-α, injected every 2–3 days at 25,000 U/mouse, a significant inhibitory effect on in vivo tumor growth was observed and was even more pronounced than that observed with in vitro preactivated CTL (data not shown). This effect was significantly inhibited in the presence of rhTRAILR2-Fc used at 70 μg/mouse/injection,

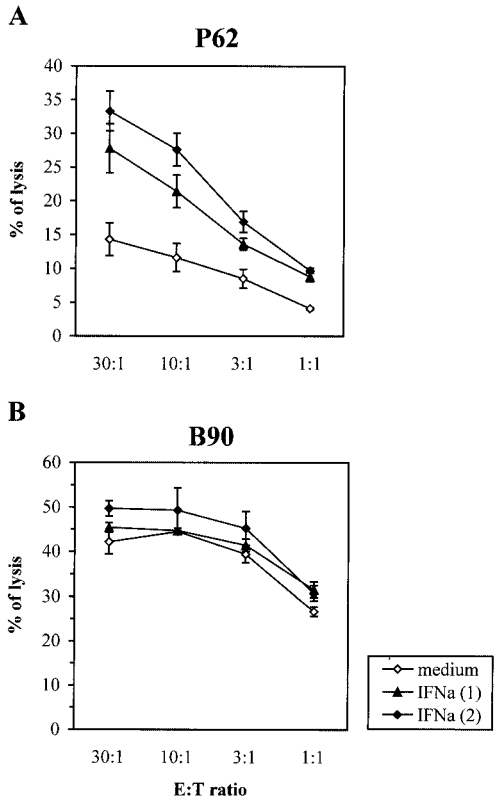

FIGURE 4. Effect of IFN-α on lytic activity of CD4$^+$ (*A*) and CD8$^+$ (*B*) CTL clones toward IGR-Pub and IGR-B2 autologous tumor cell lines. Cytotoxicity experiments were performed either in medium or after preincubating effector cells for 2 h with IFN-α (200 U/ml), followed (1) or not (2) by subsequent washing before their addition to the microtiter wells. Data represent mean ± SD of triplicates.

strongly suggesting that tumor growth inhibition mediated by the CTL clone was APO2L/TRAIL dependent (Fig. 6).

Discussion

The TNF family of molecules is a growing group of cytokines that exert a variety of effects on different cells (37). So far the most known function of these molecules is the induction of cell death upon binding to their cognate receptors. Although TNF and FasL have potent cytotoxic activity toward many tumor cells, the application of these death ligands to cancer therapy has been restricted by their severe toxicity to normal cells (3, 4). The discovery of APO2L/TRAIL as a death ligand has suggested that it might be more appropriate than TNF of FasL for systemic cancer therapy (5, 6). Expression regulation of APO2L/TRAIL on human T cells was not, so far, extensively explored and controversial results have been reported. Indeed, it has been described that APO2L/TRAIL was not detected on the surface of fresh human CD4$^+$ and CD8$^+$ PBL activated with anti-CD3 mAb, unless IFN-α was added (31). In contrast, it has been reported by the same group that APO2L/TRAIL expression was constitutive in human CD4$^+$ T cell clones and did not change upon CD3 stimulation (26). A third pattern of APO2L/TRAIL expression has been described in human mixed T cell blast populations undergoing activation-induced cell death (38). In the latter study, APO2L/TRAIL was mainly localized intracellularly in lysosomal-like compartments and upon an additional stimulation, such as PHA or anti-CD59 activation, it was secreted on the surface of internal microvesicles. In the present report, we have investigated the expression and function of APO2L/TRAIL in human CD4$^+$ and CD8$^+$ CTL clones derived from TIL. For this purpose, we have established and characterized two APO2L/TRAIL-sensitive lung carcinoma cell lines and isolated from autologous TIL CD4$^+$- and CD8$^+$-specific CTL clones. These clones expressed a CD3$^+$CD28$^-$CCR7$^-$CD45RA$^-$CD45RO$^+$ phenotype and mediated a specific HLA-restricted cytotoxicity toward autologous tumor cells.

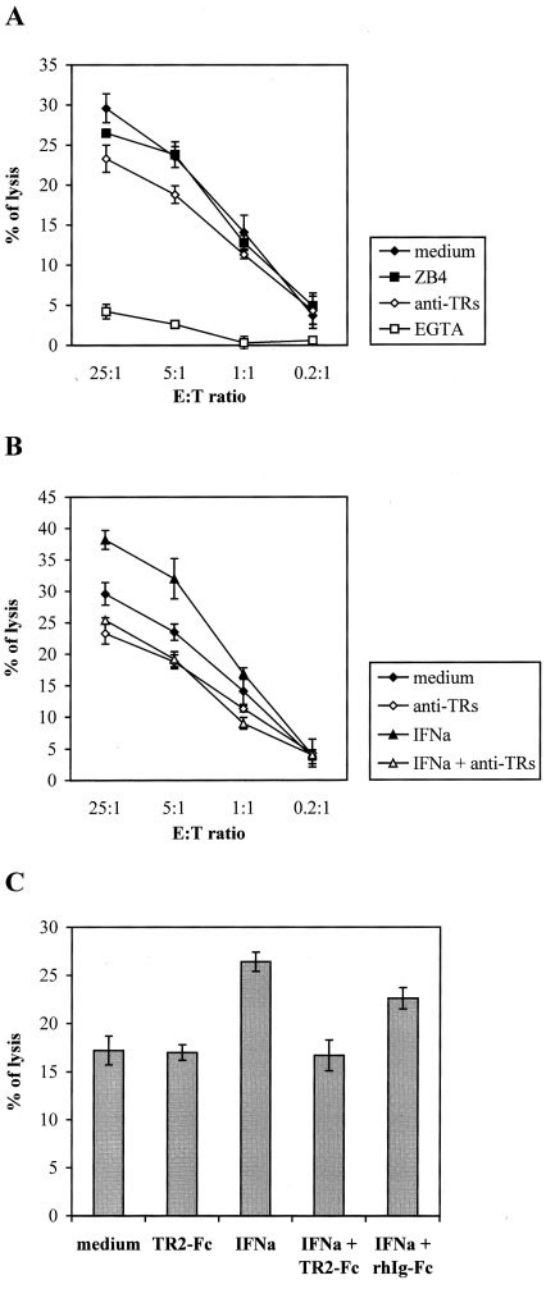

FIGURE 5. Implication of APO2L/TRAIL pathway in CD4$^+$ CTL clone lysis toward autologous tumor cells. *A*, Cytotoxic activity of the P62 CTL clone against IGR-Pub tumor cells was determined by conventional 6-h ^{51}Cr release assay performed after preincubation of target cells for 2 h either in medium or in the presence of antagonistic anti-Fas (ZB4) or anti-TRAIL-R (TRAIL-R1 plus TRAIL-R2) mAbs, or after addition of EGTA and MgCl2 during the assay. *B*, Inhibition of APO2L/TRAIL pathway was performed by preincubating IGR-Pub target cells for 2 h with anti-TRAIL-R1 plus anti-TRAIL-R2 mAbs. P62 CTL were then added at indicated E:T ratios for 6-h incubation. Experiments were performed either in medium or in the presence of IFN-α (200 U/ml) added during the cytotoxicity assay. *C*, Blocking effect of rhTRAILR2-Fc molecule or an irrelevant Fc-fusion protein (rhIg-Fc) on P62 effector cells was analyzed by their addition at 10 μg/ml concentration during the assay in the presence or absence of IFN-α (200 U/ml). The E:T ratio was 20:1. Data represent mean ± SD of triplicates.

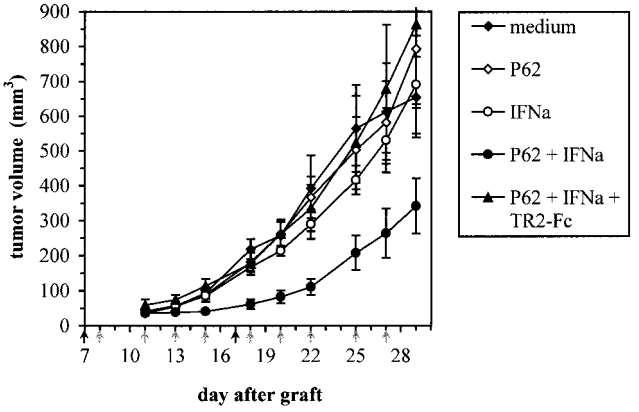

FIGURE 6. Effect of IFN-α on in vivo P62 CD4$^+$ CTL antitumor activity against IGR-Pub tumors engrafted into SCID/NOD mice. Mice were engrafted with tumor and then injected intratumorally on days 7 and 17 (black arrows) with 5×10^6 P62 CTL/mouse or control medium. IFN-α (25,000 U/mouse/injection) or IFN-α + rhTRAILR2-Fc (25,000 U + 70 μg/mouse/injection) were then injected intratumorally every 2–3 days (gray arrows). One representative experiment is shown. Tumor volumes are given as mean (±SEM) of five mice per group.

Our results demonstrated that like FasL, APO2L/TRAIL was not constitutively expressed on the surface of Ag-specific CD4$^+$ and CD8$^+$ CTL clones. Study reported by Kayagaki et al. (26) showed that expression of APO2L/TRAIL on human CD4$^+$ T cell clones established from PHA blasts was constitutive. This discrepancy may be related to the basal stimulation status of the T cell clones with feeder cells, PHA and/or IL2. In our particular model, APO2L/TRAIL surface expression was investigated 3 wk following T cell clone activation with autologous tumor cells and without previous stimulation with IL-2. More importantly, our data indicate that, while CD4$^+$ CTL clone strongly expressed APO2L/TRAIL upon specific tumor stimulation, with a slight increase in the presence of IFN-α, the CD8$^+$ clone only transiently expressed low levels of APO2L/TRAIL following IFN-α stimulation even in the absence of TCR engagement. Furthermore, our findings suggest that specific activation of CD4$^+$ CTL by an autologous target is required for a sustained IFN-α-induced APO2L/TRAIL expression increase. These results suggest a differential APO2L/TRAIL expression regulation on CD4$^+$ and CD8$^+$ CTL clones used in this report. Studies performed by Kayagaki et al. (31) showed similar APO2L/TRAIL expression on CD4$^+$ and CD8$^+$ peripheral blood T lymphocytes upon stimulation with anti-CD3 mAb and IFN-α. These expression profiles may be characteristics of the T cells analyzed (TIL vs PBL) and/or may result from the nature of the stimulus used (anti-CD3 mAb vs specific tumor target cells). However, a slight APO2L/TRAIL expression increase was observed on B90 CD8$^+$ CTL following anti-CD3 stimulation in the presence of IFN-α but never reached that observed on CD4$^+$ T cell clone (data not shown).

Further studies aimed to determine the involvement of APO2L/TRAIL in the HLA-restricted CD4$^+$ and CD8$^+$ CTL clone-mediated lysis toward their specific tumor targets. Our data indicated that as opposed to the CD8$^+$ CTL clone, which seemed to exclusively involve the granule exocytosis pathway to kill its specific target, the CD4$^+$ clone used both perforin/granzymes and APO2L/TRAIL pathways to lyse the autologous tumor cells. Furthermore, CD4$^+$ CTL-mediated target killing was significantly enhanced in the presence of IFN-α, at least by a mechanism involving APO2L/TRAIL. These results indicate that part of CD4$^+$ TCR-mediated specific cytotoxicity is APO2L/TRAIL-mediated and correlates with previous studies on TCR-independent MHC-unrestricted CD4$^+$ T cell clone cytotoxicity (26). The absence of killing of K562 and allogeneic NSCLC tumor cell lines by CD4$^+$ CTL, even though they were sensitive to APO2L/TRAIL-dependent apoptosis, further supports the finding that TCR triggering is needed to induce APO2L/TRAIL expression and its subsequent involvement in T cell-mediated tumor lysis.

The Ag specificity of T cell-mediated cytotoxicity is a favorable feature able to selectively eliminate the Ag-presenting targets, such as tumor cells, without damaging bystander non-APCs. It has been previously demonstrated that perforin/granzyme-mediated lysis is highly Ag-specific, while FasL and APO2L/TRAIL-mediated killing participate in both Ag-specific and bystander cytotoxicities (26, 39). Furthermore, previous studies showed that APO2L/TRAIL, at least partially, mediated Ag-specific cytotoxicity of a human CD4$^+$ T cell clone against FasL-resistant melanoma cells (25). The present study supplements APO2L/TRAIL as an effector molecule used by CD4$^+$ TIL to lyse autologous tumor cells, following stimulation with specific Ag, in a TCR-dependent MHC-class II-restricted manner. In addition, even though the IGR-Pub NSCLC tumor cell line expressed CD95 and was sensitive to Fas-mediated lysis (data not shown), the autologous P62 CD4$^+$ T cell clone preferentially used perforin/granzymes and APO2L/TRAIL pathways to kill its specific target. Therefore, it is possible that APO2L/TRAIL-dependent cytotoxicity mediated by the CD4$^+$ CTL clone to kill its specific tumor cells might play a major role in cancer control. Indeed, it has been previously reported that NSCLC tumors are often infiltrated by activated/memory CD4$^+$ T lymphocytes (40–42) and tumor Ag-specific CD4$^+$ CTL clones have been identified in melanoma (43–46). On the basis of these observations, we may now reconsider the potential role of the CD4$^+$ CTL subset in tumor surveillance at least for MHC class II$^+$ tumors.

Although the physiological and pathological roles of APO2L/TRAIL remain to be clearly established, it has been proposed to correspond to a potential cancer therapeutic agent. Indeed, APO2L/TRAIL has been described to induce apoptosis in a wide variety of transformed cell lines, but not in most normal cells (1, 2, 5, 6, 14, 15). Furthermore, i.p. or i.v. injection of soluble APO2L/TRAIL suppressed tumor growth in mice and nonhuman primates without being toxic to normal tissues, and chemotherapeutic drugs synergistically suppressed tumor growth in SCID mice (5, 6, 47). APO2L/TRAIL was also successfully used in locoregional treatment of glioblastoma xenografts in athymic mice (48). In our particular model, based on the use of SCID/NOD mice engrafted with human NSCLC tumors and transplanted with autologous CD4$^+$ CTL (APO2L/TRAIL$^-$), administration of IFN-α, used for the first time in vivo to explore the effect of this cytokine on human APO2L/TRAIL pathway, led to significant tumor growth suppression. This argues that APO2L/TRAIL-mediated T cell cytotoxicity may play a role in tumor cell surveillance, as supported by the rhTRAILR2-Fc inhibitory effect. However, one may note that as opposed to the in vitro studies, IFN-α seemed necessary to induce APO2L/TRAIL expression on effector cells in vivo. The apparent absence of correlation between in vivo and in vitro studies, in terms of induction of APO2L/TRAIL expression on the CD4$^+$ T cells following stimulation with the autologous tumor cells, may be attributed to the effective E:T ratios occurring in vivo, as compared with the aphysiological E:T ratios used in vitro. Alternatively, it may be correlated to the in vivo effects of IFN-α which may synergize with autologous tumor cells to induce stronger APO2L/TRAIL expression on the effector cells and earlier than in vitro. Discrepancies between in

vivo and in vitro studies have been previously reported in the CD95 (APO-1/Fas)-induced apoptosis pathway (49). Indeed, it has been reported that cellular FLIP-overexpressing tumors escape from T cell immunity in vivo despite the fact that they are efficiently killed in vitro. It has also been suggested that this apparent discrepancy was most likely due to limitations of in vitro assays, which do not accurately reflect the microenvironment in the tumor (49). In conclusion, our data emphasize a therapeutic strategy based on intratumoral IFN-α injection and/or local administration of soluble APO2L/TRAIL as a valuable treatment of lung carcinoma or as an adjuvant in future vaccination approaches. Further studies on APO2L/TRAIL expression regulation on specific T cells will provide new insights into physiological and pathological roles of APO2L/TRAIL-induced apoptosis and its potential application in cancer immunotherapy.

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