Type I Interferons (IFNs) Regulate Tumor Necrosis Factor–related Apoptosis-inducing Ligand (TRAIL) Expression on Human T Cells: A Novel Mechanism for the Antitumor Effects of Type I IFNs

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Summary

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a proapoptotic member of the TNF family of type II membrane proteins, which constitutes one component of T cell cytotoxicity. In this study, we investigated the expression and function of TRAIL in human peripheral blood T (PBT) cells. Although freshly isolated PBT cells did not express a detectable level of TRAIL on their surface, a remarkable TRAIL expression was rapidly induced on the surface of both $CD4^+$ and $CD8^+$ PBT cells upon stimulation with anti-CD3 monoclonal antibody and type I interferons (IFNs). This enhancement of TRAIL expression was a unique feature of type I IFNs (IFN-α and IFN-β), and neither type II IFN (IFN-γ) nor various other cytokines enhanced TRAIL expression on anti-CD3–stimulated PBT cells. Type I IFNs have been used for clinical treatment of renal cell carcinomas (RCCs), and we found that most RCC cell lines were susceptible to TRAIL-induced apoptosis. Type I IFNs substantially augmented cytotoxic activity of anti-CD3–stimulated PBT cells against RCC cell lines in a TRAIL-dependent manner. These results indicate a unique feature of type I IFNs to regulate TRAIL-mediated T cell cytotoxicity, which may be involved in the antitumor effects of type I IFNs against various tumors.

Key words: cytotoxic T lymphocyte • cytotoxicity • TRAIL • type I interferon • renal cell carcinoma

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)[1]/APO-2L is a recently identified type II integral membrane protein belonging to the TNF family, which induces apoptotic cell death in a variety of tumor cells (1–3). Among the members of this family, TRAIL exhibits highest homology to a well-characterized proapoptotic factor, FasL/CD95L, which has been implicated in T cell cytotoxicity and immune regulation (4). TRAIL mRNA was found in a variety of tissues and cells (1), but little is known about the expression of TRAIL at the protein level or its physiological functions. Recent studies revealed that at least four receptors, TRAIL-R1/DR4, TRAIL-R2/DR5/TRICK2/killer, TRAIL-R3/TRID/DcR1/LIT, and TRAIL-R4/TRUNDD/DcR2, bind to TRAIL with similar affinities (5–10). Both TRAIL-R1 and TRAIL-R2 contain a death domain homologous to that in Fas, TNFR-I, and DR3/TRAMP/APO-3/WSL-1. Oligomerization of the death domain in TRAIL-R1 and TRAIL-R2 recruits caspase-8 or -10 via Fas-associated death domain (FADD) or a FADD-like adapter molecule, and activates the subsequent caspase cascade resulting in apoptotic cell death (5–8, 10). In contrast to these proapoptotic receptors, TRAIL-R3 completely lacks a cytoplasmic domain and exists as a glycophospholipid-anchored protein on the cell surface. Because TRAIL-R3 can compete with the proapoptotic TRAIL-Rs for TRAIL binding, it seems to work as a decoy receptor. It has been suggested that TRAIL in-

[1]*Abbreviations used in this paper:* CMA, concanamycin A; CML, chronic myelogenous leukemia; PBT, peripheral blood T; poly IC, polyinosinic acid:polycytidylic acid; RCC, renal cell carcinoma; STAT, signal transducer and activator of transcription; TIL, tumor-infiltrating T lymphocyte; TRAIL, TNF-related apoptosis-inducing ligand.

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duces apoptosis preferentially in transformed cells but not in normal cells, possibly due to preferential expression of TRAIL-R3 in the latter (6, 8, 10). TRAIL-R4 carries a cytoplasmic domain containing a truncated death domain that cannot transmit a death signal but can activate nuclear factor κB, which may protect the cells from TRAIL-R1– or TRAIL-R2–induced apoptosis (9, 10). To date, the physiological functions of these multiple TRAIL-Rs remain obscure.

It has been shown that CTLs kill target cells by two major effector mechanisms, which are mediated by perforin or FasL (11–13), and that these effector molecules are required for the elimination of virus-infected cells or certain tumor cells (14–16). Furthermore, we and others have demonstrated that these molecules are responsible for mediating tissue damage associated with T cell–mediated diseases such as graft-versus-host disease and hepatitis by using murine models (17–19). In addition to these molecules, we recently revealed that TRAIL was expressed on human $CD4^+$ T cell clones and mediated their cytotoxicity against some tumor cells, such as a human T lymphoma Jurkat and a transformed keratinocyte HaCaT (20). Thomas and Hersey also reported recently that TRAIL at least partly mediated cytotoxicity of melanoma-specific $CD4^+$ T cell clones against some melanoma cells and Jurkat cells (3). These observations suggested that TRAIL constitutes an additional pathway of T cell cytotoxicity against certain tumor cells. However, it remains to be determined whether TRAIL can be expressed not only on particular T cell clones but also on circulating T cells and whether it can be used as a general mechanism of T cell cytotoxicity.

IFNs are the most classical cytokines, and were discovered on the basis of their antiviral activities. It is well known that a variety of cells produce IFNs in response to infection by various viruses, bacteria, and mycoplasmas (21, 22). IFNs can be classified into two groups, type I (IFN-α, IFN-β, and IFN-ω) and type II (IFN-γ). All of the type I IFNs share the same cell surface receptor (IFNAR) composed of at least two subunits, IFNAR-1 and IFNAR-2b/c. In addition to their antiviral effects, IFNs exhibit pleiotropic biological activities, including antitumor and immunomodulatory effects (21, 22). Type I IFNs have been commonly used for clinical treatment of certain tumors, including melanomas, gliomas, chronic myelogenous leukemias (CMLs), and renal cell carcinomas (RCCs) (23, 24). The antitumor effects of type I IFNs appear to be mediated by either or both of the following two mechanisms: (a) direct cytotoxic or cytostatic activity against tumor cells, and (b) indirect cytotoxicity by modulating host immune responses. However, the effector molecules responsible for mediating the antitumor effects of type I IFNs remain largely unknown.

In this study, we characterized the expression and function of TRAIL on human peripheral blood T (PBT) cells, and found a unique feature of type I IFNs to upregulate TRAIL expression on anti-CD3–stimulated PBT cells. We also demonstrated that almost all RCC cell lines are susceptible to TRAIL-induced cytotoxicity, and that type I IFNs augment cytotoxic activity of anti-CD3–stimulated PBT cells against RCC cell lines in a TRAIL-dependent manner. The physiological and clinical relevance of these findings is discussed.

Materials and Methods

Cells. A human T lymphoma cell line Jurkat was obtained from Japan Cancer Research Bank and cultured in RPMI 1640 medium containing 10% FCS, 100 μg/ml streptomycin and penicillin, and 2 mM glutamine (culture medium). A mouse B lymphoma cell line 2PK-3 was obtained from American Type Culture Collection and maintained in culture medium. 2PK-3–derived transfectants, hTRAIL/2PK-3 and hFasL/2PK-3, which stably express human TRAIL and human FasL, respectively, were prepared as described previously (20) and maintained in culture medium. Human RCC-derived cell lines 769P, A498, ACHN, Caki 1, and Caki 2 were obtained from American Type Culture Collection, and KO-RCC-1 was established as described previously (25). RCC-derived cell lines KN-39, KN-41, and OWR-10 were provided by Dr. Y. Kinoshita (Kobe University, Kobe, Japan). All of these RCC-derived cell lines were maintained in culture medium.

Reagents. Human IFN-γ, TNF-α, IL-4, IL-6, IL-15, and IL-16 were purchased from PharMingen. Human IL-1α, IL-1β, and IL-7 were purchased from Genzyme. Human IL-12 was purchased from R&D Systems. Human IL-2, IFN-α, and IFN-β were provided by Shionogi (Osaka, Japan), Sumitomo Pharma (Osaka, Japan), and Toray (Kamakura, Japan), respectively. Human IL-18 was provided by Drs. H. Tsutsui, H. Okamura, and K. Nakanishi (Hyogo College of Medicine, Nishinomiya, Japan). A synthetic double-stranded RNA, polyinosinic acid:polycytidylic acid (poly IC), and PHA were purchased from Sigma. Concanamycin A (CMA), which inhibits perforin-mediated cytotoxicity (26), was purchased from Wako Pure Chemicals. An anti-CD28 mAb (CD28.2) was purchased from PharMingen. An anti-CD3 mAb, OKT-3, was prepared from the hybridoma obtained from American Type Culture Collection. A neutralizing anti–human FasL mAb (NOK-2, mouse IgG2a/κ) and a neutralizing anti–human TRAIL mAb (RIK-2, mouse IgG1/κ) were prepared as described previously (20, 27).

Cell Preparation and Activation. PBMCs were prepared from healthy volunteers by Ficoll-Hypaque (Sigma) centrifugation. PBMCs (3×10^6 cells/ml) were cultured at 37°C for 24 or 48 h on 24-well plates precoated with 10 μg/ml of anti-CD3 mAb, or in the presence of IL-2 (500 U/ml), PHA (20 μg/ml), or poly IC (20 μg/ml).

For preparation of PBT cells, PBMCs were first cultured on plastic dishes for 1 h at 37°C to deplete adherent monocytes. After passage of nonadherent cells through a nylon wool column, PBT cells were isolated by E-rosetting with sheep red blood cells followed by depletion of residual NK and B cells by using anti-CD16 mAb (3G8; PharMingen), anti-CD20 mAb (2H7; PharMingen), anti-CD56 mAb (B159; PharMingen), and anti–mouse Ig immunomagnetic beads (Dynal). Purity of PBT cells was >95% $CD3^+$ as determined by flow cytometry. In some experiments, $CD4^+$ and $CD8^+$ T cells were isolated from PBT cells by using anti-CD4 or anti-CD8 immunomagnetic beads (Dynal) and DetachaBead® (Dynal). The purity of each population was >98% $CD3^+CD4^+$ or >98% $CD3^+CD8^+$ as estimated by flow cytometry. Purified PBT cells (3×10^6 cells/ml) were cultured on plates uncoated or precoated with anti-CD3 mAb (10 μg/ml) in the presence or absence of IFN-α (200 U/ml), IFN-β (200 U/ml), IFN-γ (500 U/ml), TNF-α (100 ng/ml), IL-1α (50 ng/ml), IL-1β

(50 ng/ml), IL-2 (500 U/ml), IL-4 (50 ng/ml), IL-6 (200 U/ml), IL-7 (20 ng/ml), IL-12 (20 ng/ml), IL-15 (150 ng/ml), IL-16 (100 ng/ml), or IL-18 (500 ng/ml) at 37°C for 2 or 48 h. In some experiments, soluble anti-CD28 mAb (10 μg/ml) was added to the culture.

Flow Cytometric Analysis. Cells (10^6) were incubated with 1 μg of biotinylated RIK-2 or control mouse IgG1 (PharMingen) for 1 h at 4°C followed by PE-labeled avidin (PharMingen). After washing with PBS, the cells were analyzed on a FACScan™ (Becton Dickinson), and data were processed by using Cell Quest™ software (Becton Dickinson). In some experiments, FITC-labeled anti–human CD3 mAb (PharMingen) was included to identify T cells in PBMCs.

Northern Blot Analysis. PBT cells (3×10^6 cells/ml) were cultured on plates uncoated or precoated with anti-CD3 mAb (10 μg/ml) in the presence or absence of IFN-α (200 U/ml) for 12 h. Total RNA was then extracted from the cells by using RNA STAT-60™ (Tel-test, Inc.) according to the manufacturer's instructions. 10 μg each of denatured RNAs was electrophoresed in a 1.5% agarose gel containing 6.6% formaldehyde, and then transferred to a nylon membrane (Pall). The membrane was hybridized for 4 h with an [α-^{32}P]dCTP-labeled 900-bp XhoI-NotI fragment containing human TRAIL cDNA (20) at 65°C in ExpressHyb™ hybridization solution (Clontech), and then washed twice in 2× SSC/0.1% SDS at 65°C for 15 min and twice in 0.5× SSC/0.1% SDS at 65°C for 15 min. The membrane was analyzed by autoradiography (BAS2500; Fuji Film, Inc.). In a similar way, the same membrane was rehybridized with a 1-kb EcoRI-BamHI fragment containing β-actin cDNA (28).

Cytotoxic Assay. PBT cells (3×10^6 cells/ml) were prestimulated for 12 h on plates uncoated or precoated with anti-CD3 mAb (10 μg/ml) in the presence or absence of IFN-α (200 U/ml). After washing twice with culture medium, the cells were used as effector cells. In some experiments, human TRAIL or FasL transfectants were used as effector cells.

A ^{51}Cr-release assay was performed as described previously (29). In brief, ^{51}Cr-labeled target cells (10^4) and effector cells were mixed in U-bottomed wells of a 96-well microtiter plate at the indicated E/T ratios. After an 8-h incubation, cell-free supernatants were collected and radioactivity was measured in a γ-counter. Percent specific ^{51}Cr release was calculated as described (29). In some experiments, the effector cells were pretreated with 20 nM CMA for 2 h to inactivate perforin (26). Anti-FasL mAb (NOK-2) and/or anti-TRAIL mAb (RIK-2) were added to a final concentration of 10 μg/ml each at the start of the cytotoxic assay.

Results

Expression of TRAIL on Anti-CD3– and IFN-α–stimulated PBT Cells. We recently demonstrated that human CD4$^+$ T cell clones constitutively expressed TRAIL on their surface as estimated by staining with neutralizing anti-TRAIL mAbs, RIK-1 and RIK-2 (20). In contrast, as represented in Fig. 1, we could not find a detectable level of surface TRAIL on freshly isolated PBT cells. Because the expression of TNF family proteins such as TNF-α and FasL can be induced on activated T cells, we examined whether surface TRAIL expression can be also upregulated on PBT cells by various stimuli. PBMCs were stimulated with immobilized anti-CD3 mAb, PHA, IL-2, or poly IC for 24 or 48 h, and TRAIL expression on CD3$^+$ PBT cells was then examined by flow cytometry with RIK-2 mAb. As represented in Fig. 1, no detectable level of TRAIL was found on PBT cells when stimulated with PHA or IL-2. In contrast, marginal but significant levels of TRAIL expression were consistently observed upon stimulation with anti-CD3 mAb or poly IC.

Since poly IC has been known to be a potent inducer of type I IFNs (21, 22), we next examined whether IFN-α can directly affect TRAIL expression on PBT cells. As represented in Fig. 2 A, marginal but significant levels of TRAIL expression were observed on both CD4$^+$ and CD8$^+$ PBT cells at 12 h after stimulation with either anti-CD3 mAb or IFN-α alone. Interestingly, the combination of anti-CD3 mAb and IFN-α greatly upregulated TRAIL expression on both CD4$^+$ and CD8$^+$ PBT cells. Similar results were obtained at 24 or 48 h after each stimulation (data not shown). These results indicated that stimulation with anti-CD3 mAb alone is not sufficient for maximal induction of surface TRAIL expression on PBT cells, and that IFN-α provides a costimulatory signal that synergistically upregulates TRAIL expression.

It has been shown that expression of some TNF family proteins such as CD40L on activated T cells was upregu-

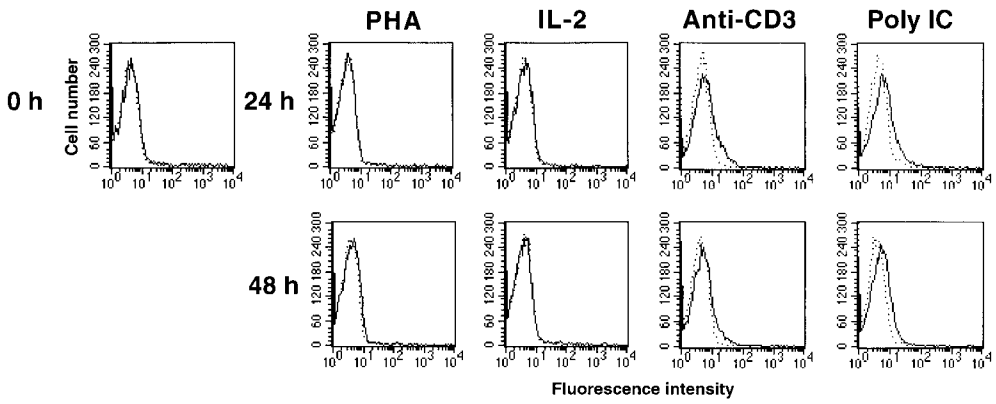

Figure 1. Expression of TRAIL on human PBT cells. PBMCs were cultured for the indicated time periods on plates precoated with anti-CD3 mAb (10 μg/ml) or in the presence of PHA (20 μg/ml), IL-2 (500 U/ml), or poly IC (20 μg/ml). The cells were then stained with biotinylated RIK-2 followed by PE-labeled avidin and FITC-labeled anti-CD3 mAb. Solid lines, staining with RIK-2 on CD3$^+$ PBT cells. Dotted lines, background staining with biotinylated control IgG and PE-labeled avidin. Similar results were obtained with PBT cells from three individuals.

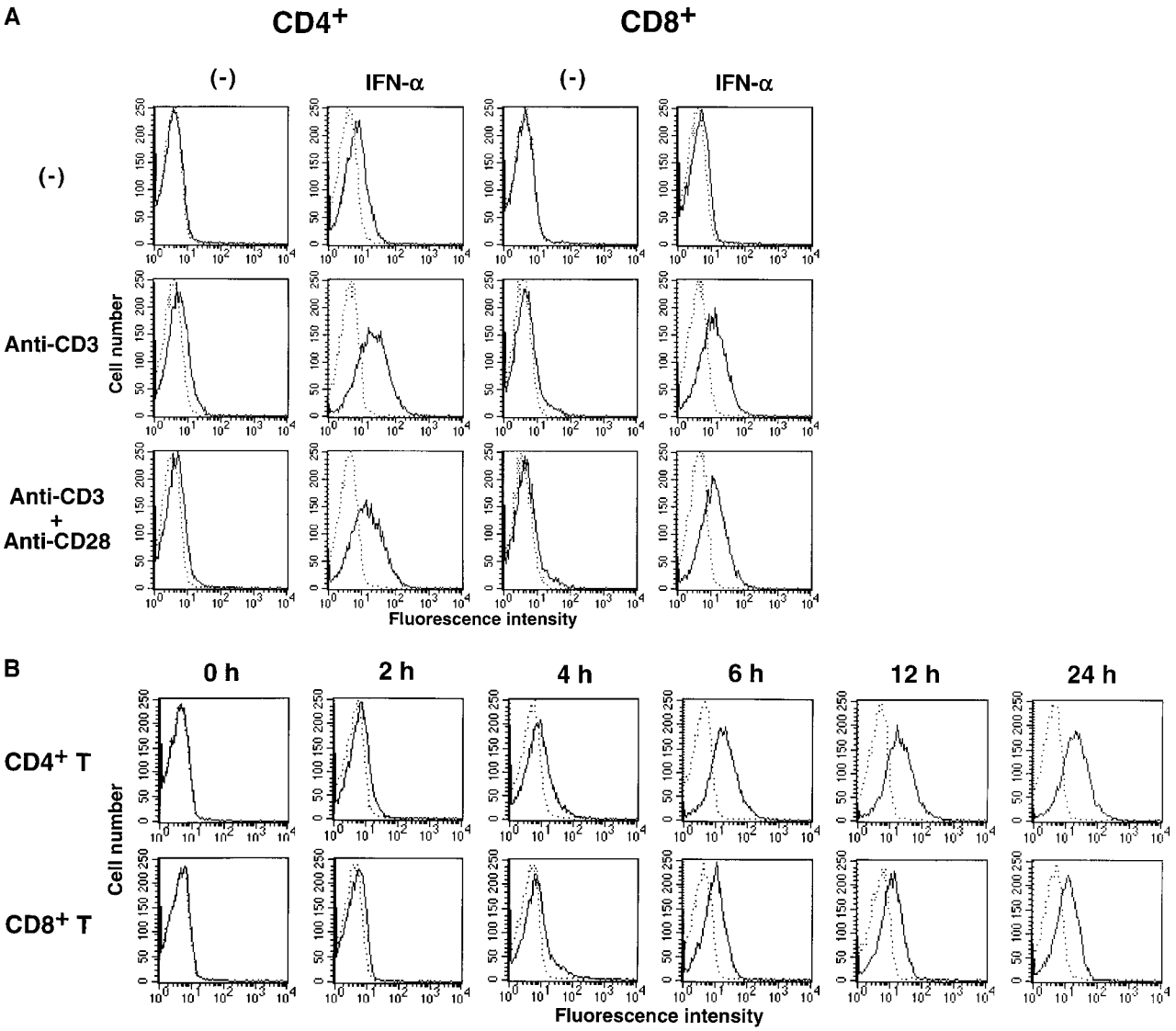

Figure 2. Regulation of surface TRAIL expression on human $CD4^+$ or $CD8^+$ PBT cells by TCR/CD3 and/or IFN-α. (A) Purified $CD4^+$ or $CD8^+$ PBT cells were cultured for 12 h on plates uncoated or precoated with anti-CD3 mAb (10 μg/ml) in the presence or absence of IFN-α (200 U/ml) and/or soluble anti-CD28 mAb (10 μg/ml), and then stained with biotinylated RIK-2 and PE-avidin (solid lines). Dotted lines, background staining with biotinylated control IgG and PE-avidin. Similar results were obtained with $CD4^+$ or $CD8^+$ T cells from three individuals. (B) Time course of TRAIL expression on human $CD4^+$ or $CD8^+$ PBT cells after stimulation with anti–CD3 plus IFN-α. $CD4^+$ or $CD8^+$ PBT cells were cultured on plates precoated with anti-CD3 mAb (10 μg/ml) in the presence of IFN-α (200 U/ml). After the indicated time periods, the cells were stained with biotinylated RIK-2 and PE-avidin (solid lines). Dotted lines, background staining with biotinylated control IgG and PE-avidin. Similar results were obtained with $CD4^+$ or $CD8^+$ T cells from three individuals.

lated by a CD28-mediated costimulatory signal (30). Therefore, we tested whether the CD28-mediated costimulation might affect expression of TRAIL on anti-CD3–stimulated PBT cells. As represented in Fig. 2 A, the addition of an agonistic anti-CD28 mAb barely affected the expression levels of surface TRAIL on anti-CD3– or anti-CD3 plus IFN-α–stimulated PBT cells, indicating that the CD28-mediated costimulatory signal does not contribute to TRAIL induction in PBT cells.

We also examined the kinetics of TRAIL expression after stimulation with anti-CD3 plus IFN-α. As represented in Fig. 2 B, TRAIL expression appeared on both $CD4^+$ and $CD8^+$ PBT cells at 2 h after stimulation, and reached a peak at 6 h. Similar levels of surface TRAIL expression were retained on $CD4^+$ and $CD8^+$ PBT cells at 48 h (not shown).

Characterization of Cytokines that Can Regulate TRAIL Expression. We next examined various cytokines for their ability to regulate TRAIL expression on unstimulated or anti-CD3–stimulated PBT cells. IFN-β, another type I IFN that shares the same receptor (IFNAR) with IFN-α (21, 22), exhibited a similar effect as IFN-α, which alone induced a marginal TRAIL expression on PBT cells and greatly upregulated TRAIL expression on anti-CD3–stimulated PBT cells (Fig. 3). Similar levels of TRAIL expres-

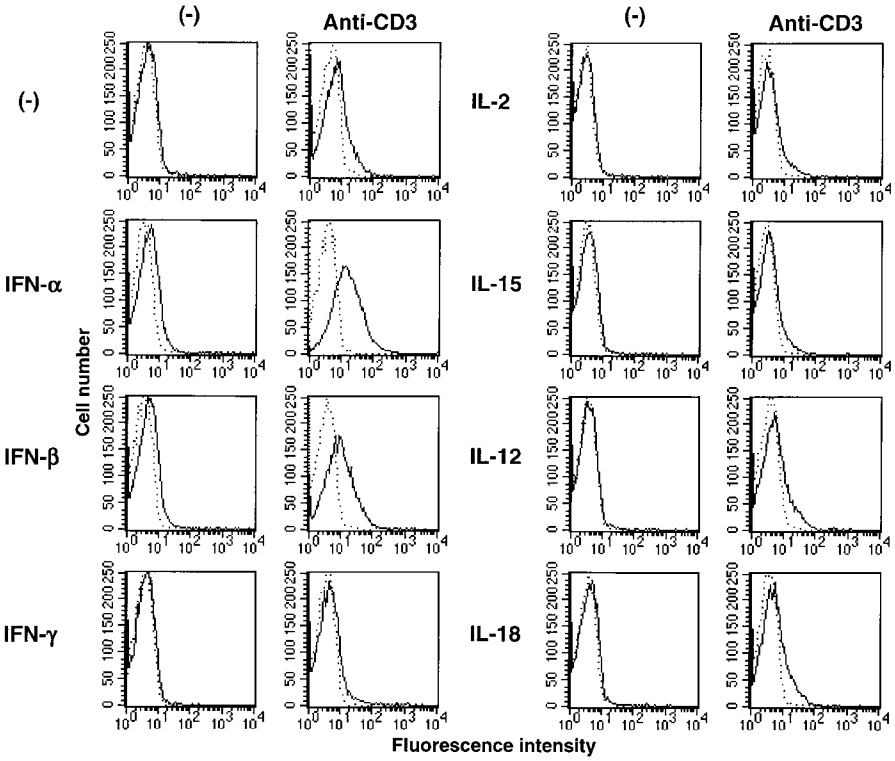

Figure 3. Regulation of TRAIL expression on human PBT cells by various cytokines. PBT cells were cultured for 12 h on plates uncoated or precoated with anti-CD3 mAb (10 μg/ml) in the presence or absence of IFN-α (200 U/ml), IFN-β (200 U/ml), IFN-γ (500 U/ml), IL-2 (500 U/ml), IL-12 (20 ng/ml), IL-15 (150 ng/ml), or IL-18 (500 ng/ml). The cells were then stained with biotinylated RIK-2 and PE-avidin (solid lines). Dotted lines, background staining with biotinylated control IgG and PE-avidin. Similar results were obtained with PBT cells from three individuals.

sion on anti-CD3–stimulated PBT cells were observed upon costimulation with a lower dose of IFN-α and IFN-β (50 U/ml; not shown). In contrast, the type II IFN (IFN-γ), which stimulates a distinct receptor (IFNGR), exhibited no effect on TRAIL expression on unstimulated or anti-CD3–stimulated PBT cells at 12 h (Fig. 3) or 24 or 48 h (not shown) even at a high dose of 500 U/ml. We also examined IL-2, IL-15, IL-12, and IL-18, which have been reported to upregulate FasL expression on T and NK cells (4, 31–33), but observed no effect on TRAIL expression (Fig. 3). Similarly, no effect was observed with the other cytokines tested, including TNF-α, IL-1α, IL-1β, IL-4, IL-6, IL-7, and IL-16 (data not shown). These results indicated that the ability to induce TRAIL expression on PBT cells is a unique feature of type I IFNs.

Transcriptional Regulation of TRAIL Expression in PBT Cells by TCR/CD3 and/or Type I IFNs. It has been reported that TRAIL mRNA was detectable in a variety of tissues and cells (1). Furthermore, a recent study demonstrated TRAIL mRNA expression in human PBT cells after activation with anti-CD3 mAb or PMA plus ionomycin (34). Therefore, we examined whether stimulation with anti-CD3 mAb and/or IFN-α upregulates TRAIL expression in PBT cells at the transcriptional level. As represented in Fig. 4, PBT cells cultured with medium alone expressed only a marginal level of the 1.6-kb TRAIL transcript. Although stimulation with either IFN-α or anti-CD3 mAb alone significantly increased TRAIL mRNA expression, a remarkable increase was observed after stimulation with the combination of anti-CD3 mAb and IFN-α. It was also noted that IFN-α was more effective than anti-CD3 mAb in inducing TRAIL mRNA expression when used alone. Similar results were obtained when PBT cells were stimulated with IFN-β (not shown). These results indicated that type I IFNs regulate TRAIL expression in unstimulated or anti-CD3–stimulated PBT cells at the transcriptional level.

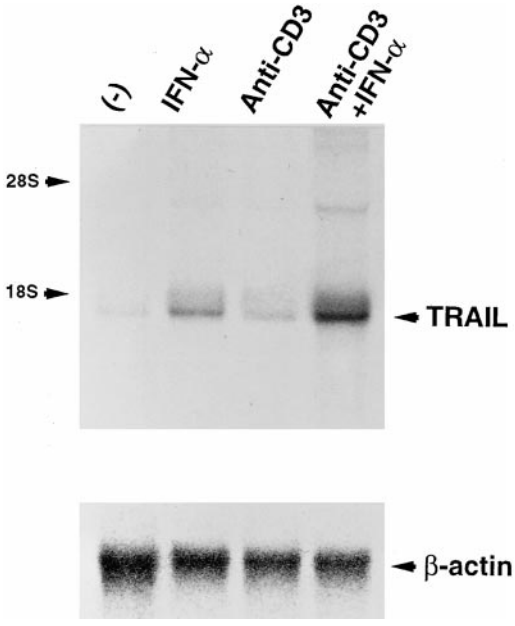

Figure 4. Northern blot analysis of TRAIL mRNA expression in PBT cells. PBT cells were cultured for 12 h with medium alone (−), IFN-α (200 U/ml), and/or immobilized anti-CD3 mAb (10 μg/ml). Total RNAs were extracted from the cells, and 10 μg each was subjected to Northern hybridization with human TRAIL cDNA probe (top). The blot was subsequently rehybridized with β-actin cDNA probe (bottom). Similar results were obtained with PBT cells from two individuals.

Involvement of TRAIL in IFN-α–enhanced T Cell Cytotoxicity against RCC Cell Lines. Type I IFNs have been used for clinical treatment of various tumors, including melanomas, gliomas, CMLs, and RCCs (23, 24). The unique feature of type I IFNs to upregulate TRAIL expression on PBT cells prompted us to examine whether IFN-induced TRAIL on PBT cells might be directly involved in enhanced cytotoxicity against tumor cells. In this respect, we first examined the susceptibility of human RCC cell lines to cytotoxicity by human TRAIL transfectants. We also examined their susceptibility to human FasL, which constitutes another pathway of T cell cytotoxicity. As represented in Fig. 5, eight out of nine RCC cell lines tested were sensitive to TRAIL-induced cytotoxicity. Some RCC cell lines (A498 and KN-41) exhibited as high sensitivity as Jurkat, which has been commonly used as a highly sensitive target for TRAIL (1–3). On the other hand, the FasL transfectants lysed five out of nine RCC cell lines, all of which were also lysed by the TRAIL transfectants. Some RCC cell lines (Caki 1, KN-39, and KN-41) were sensitive to TRAIL but not to FasL.

We then examined the cytotoxic activity of anti-CD3– and/or IFN-α–stimulated PBT cells against RCC cell lines that were sensitive to both TRAIL and FasL (ACHN and A498) or sensitive to TRAIL but resistant to FasL (KN-39 and KN-41). A neutralizing anti-TRAIL mAb (RIK-2 [20]) and a neutralizing anti-FasL mAb (NOK-2 [27]) were used to assess the contribution of TRAIL and FasL to the cytotoxicity. CMA pretreatment, which inactivates perforin (26), was used to assess the contribution of perforin. As shown in Fig. 6, anti-CD3–stimulated PBT cells exhibited substantial cytotoxic activities against both types of RCC target cells, which were partially inhibited by RIK-2 but not by NOK-2; the residual cytotoxicity was mostly abrogated by CMA treatment, indicating that these cytotoxic activities were mediated by TRAIL and perforin. Although IFN-α stimulation alone did not significantly induce cytotoxicity, costimulation with IFN-α greatly enhanced the cytotoxic activities of anti-CD3–stimulated PBT cells; this enhancement was abrogated by RIK-2 but not by NOK-2, indicating that the IFN-α–enhanced cytotoxicity was predominantly mediated by TRAIL but not by FasL. It was also noted that the TRAIL-independent residual cytotoxic activities, which were abrogated by CMA treatment, were almost comparable between the anti-CD3–stimulated T cells and the anti-CD3 plus IFN-α–stimulated T cells, indicating that IFN-α did not enhance perforin-mediated cytotoxicity. These results indicate that IFN-α can augment the cytotoxic activity of TCR/CD3-stimulated PBT cells against RCC cells by specifically enhancing TRAIL-mediated cytotoxicity.

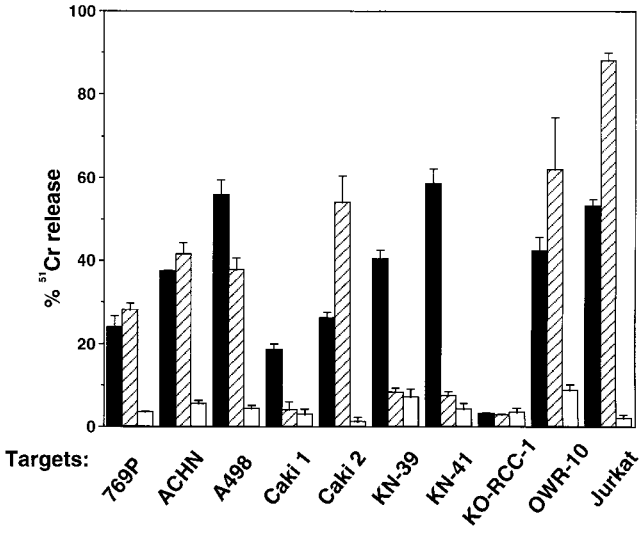

Figure 5. Cytotoxic activity of TRAIL or FasL transfectants against RCC cell lines. Cytotoxic activity of hTRAIL/2PK-3 (black bars), hFasL/2PK-3 (hatched bars), or 2PK-3 (white bars) was tested against the indicated target cells by 8 h ^{51}Cr-release assay at an E/T ratio of 10. Data represent mean ± SD of triplicate samples. Similar results were obtained in two independent experiments.

Discussion

We recently demonstrated that human CD4$^+$ T cell clones constitutively expressed TRAIL, which was fully responsible for spontaneous cytotoxicity against certain tumor cells, such as Jurkat cells (20). Others have also recently shown that the cytotoxic activity of human CD4$^+$ T cell clones against melanoma cells was at least partly mediated by TRAIL (3). In this study, we characterized the expression and function of TRAIL on human PBT cells and found that both CD4$^+$ and CD8$^+$ PBT cells express TRAIL upon TCR/CD3 stimulation, especially in the presence of type I IFNs. This indicated that TRAIL-mediated cytotoxicity is not confined to particular T cell clones but can be generally involved in TCR/CD3-mediated, antigen-specific cytotoxicity exerted by human T cells, especially when they are exposed to exogenously administrated or endogenously produced type I IFNs.

A previous study by others demonstrated the upregulation of TRAIL mRNA in human PBT cells after activation with anti-CD3 mAb or PMA plus ionomycin (34). In the present study, we demonstrated that anti-CD3 stimulation induced surface TRAIL expression on human PBT cells but only marginally. We found that IFN-α greatly enhanced TRAIL expression on anti-CD3–stimulated PBT cells, whereas IFN-α solely induced TRAIL expression on PBT cells and only marginally. This enhancement of TRAIL expression was a unique feature of type I IFNs (IFN-α and IFN-β) among the various cytokines so far tested. Other cytokines that have been shown to upregulate FasL expression in T and NK cells (IL-2, IL-12, IL-15, and IL-18 [4, 31–33]) or those enhancing T cell cytotoxicity (IL-2, IL-4, IL-6, IL-7, IL-12, IL-15, and IL-18) did not affect TRAIL expression on unstimulated or anti-CD3–stimulated PBT cells. Northern blot analysis showed that the synergistic effect of anti-CD3 and IFN-α was exerted at the transcriptional level. A higher transcriptional induction by IFN-α alone than that by anti-CD3 alone suggests the possibility that TRAIL gene expression is primarily regulated by IFN-α and that anti-CD3 stimulation may act synergistically by upregulat-

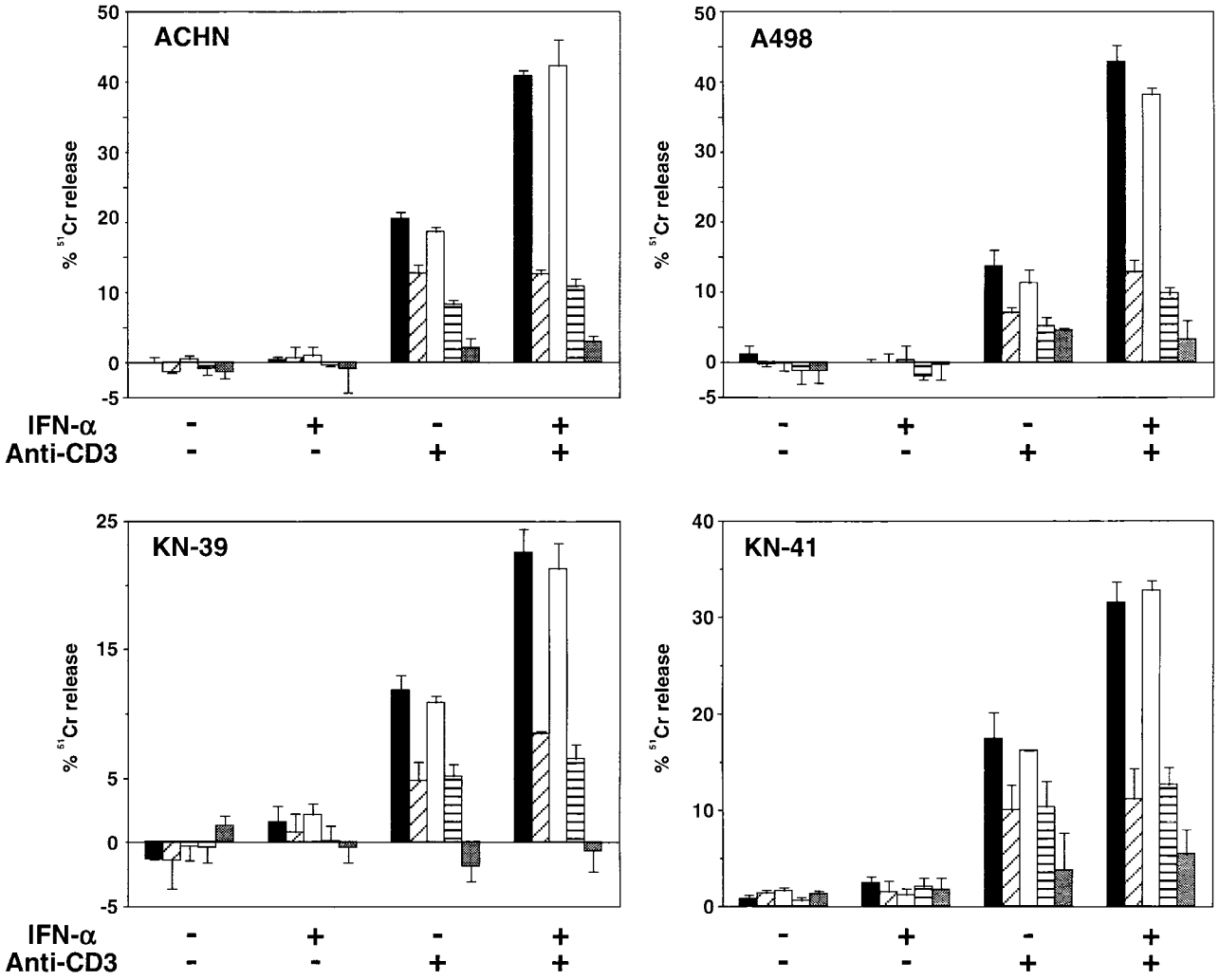

Figure 6. Involvement of TRAIL, FasL, or perforin in anti-CD3– and/or IFN-α–stimulated T cell cytotoxicity against RCC cell lines. PBT cells were stimulated with immobilized anti-CD3 mAb (10 μg/ml) and/or IFN-α (200 U/ml) for 12 h. Cytotoxic activity was then tested against ACHN, A498, KN-39, or KN-41 target cells in the presence or absence (black bars) of 10 μg/ml each of RIK-2 (alone, hatched bars), NOK-2 (alone, white bars), RIK-2 and NOK-2 (striped bars), or RIK-2 and NOK-2 with 20 nM CMA (gray bars) by 8 h ^{51}Cr-release assay at an E/T ratio of 30. Data represent mean ± SD of triplicate samples. Similar results were obtained in three independent experiments.

ing type I IFN receptors (IFNARs) on PBT cells. Binding of type I IFN to IFNAR leads to activation of the receptor-associated tyrosine kinases, Tyk-2 and Jak-1, which phosphorylate signal transducer and activator of transcription (STAT)-1 and STAT-2. Phosphorylated STAT-1 and STAT-2 form a transcriptionally active heterotrimer complex (ISGF3) together with ISGF3γ/p48, which binds to a *cis*-acting enhancer sequence termed IFN-stimulated response element (ISRE [35]). Over 30 proteins have been known to be the products of type I IFN–inducible genes whose transcription can be upregulated by ISRE in the flanking region (21, 22). Further studies are needed to characterize the regulatory region of the TRAIL gene and to elucidate the regulatory mechanisms for TRAIL expression at the transcriptional level.

Type I IFNs have been commonly used for clinical treatments of certain tumors, including melanomas, gliomas, CMLs, and RCCs (23, 24). However, the majority of these tumors are resistant to type I IFN-induced growth arrest or apoptosis in vitro. Previous studies in the mouse system have shown that the antitumor effect of type I IFNs in vivo was mediated by host T cells (36, 37). Furthermore, the combination of type I IFNs and adoptive transfer of tumor-specific T cells was effective for rejection of certain tumors (38, 39). Although it is well known that type I IFNs exert pleiotropic immunomodulatory effects including upregulation of MHC class I expression and enhancement of CTL cytotoxicity (21, 22), the effector molecules responsible for mediating the antitumor effects of type I IFNs have not been well characterized. In this study, we revealed that type I IFNs upregulate TRAIL expression on TCR/CD3-stimulated T cells and augment their cytotoxicity against TRAIL-sensitive tumor cells. We found that almost all of the RCC cell lines tested were TRAIL sensitive, and that IFN-α substantially enhanced the cytotoxic activity of TCR/CD3-stimulated PBT cells against these target cells in a TRAIL-

dependent manner. Moreover, a recent study by Thomas and Hersey demonstrated that most of the melanoma cell lines they tested were also sensitive to TRAIL-induced apoptosis (3). Therefore, it is possible that the enhancement by IFN-α of TRAIL-mediated cytotoxicity by TCR/CD3-stimulated T cells at least partly accounts for the antitumor effects of type I IFNs against RCCs and melanomas. It is known that melanomas and RCCs are highly antigenic, and tumor-infiltrating T lymphocytes (TILs) are frequently observed in patients with RCCs or melanomas (40, 41). In such a situation, the administration of type I IFNs would upregulate TRAIL expression on TCR/CD3-stimulated TILs that are reactive to some tumor antigen and thus augment their cytotoxicity against TRAIL-sensitive tumor cells. Type I IFNs would also upregulate MHC class I expression on tumor cells and thus enhance TCR/CD3-mediated upregulation of TRAIL expression on tumor-reactive T cells. In addition, it has been shown that the combination of type I IFNs and certain chemotherapeutic drugs exerts synergistic antitumor effects (23, 24). In this respect, it is noteworthy that the expression of a proapoptotic TRAIL receptor, TRAIL-R2/DR5/killer, can be upregulated in some tumor cells by chemotherapeutic drugs in a p53-dependent manner (7). Therefore, it is possible that the synergistic antitumor effects of type I IFNs and some chemotherapeutic drugs may be at least partly mediated by TRAIL induction by the former and TRAIL-R2 induction by the latter.

It is known that T cell cytotoxicity is mediated by perforin and FasL (11–13). Our present study demonstrated that TRAIL constitutes an additional predominant pathway of T cell cytotoxicity potentially against various tumor cells. However, the expression of these effector molecules in PBT cells is differently regulated. We showed previously that the expression of perforin in PBT cells is confined to a minor population of $CD8^+$ T cells that express CD11b and represent memory effector CTLs (29). We also demonstrated previously that FasL is not expressed on PBT cells but can be transiently expressed on $CD45RO^+$ memory $CD4^+$ and $CD8^+$ T cells upon stimulation with PMA plus ionomycin or anti-CD3 mAb (42). However, the cell surface expression of FasL on activated T cells is rapidly downregulated by shedding mediated by some proteases (27). Also in the present study, we examined FasL expression in anti-CD3– and/or IFN-α–stimulated PBT cells (data not shown). Rehybridization of the Northern blot shown in Fig. 4 with a human FasL cDNA probe showed that FasL mRNA expression was induced by anti-CD3 but not by IFN-α, and no enhancement of anti-CD3–induced FasL mRNA expression was observed upon costimulation with IFN-α. However, we could not find a detectable level of cell surface FasL expression on the anti-CD3–stimulated PBT cells, possibly due to the rapid shedding. Consequently, we could not find a substantial contribution of FasL to the cytotoxic activities of anti-CD3– and/or IFN-α–stimulated PBT cells even against the FasL-sensitive target cells (A498 and ACHN; Fig. 6). In contrast to this transient nature of FasL expression, TRAIL can be stably expressed on most $CD4^+$ and $CD8^+$ T cells, especially when costimulated with anti-CD3 and IFN-α (Fig. 2 B). This suggests that TRAIL can mediate more long-lasting T cell cytotoxicity than FasL, which would be beneficial for exerting an antitumor effect. In the case of FasL, the rapid downregulation by shedding has been suggested to be beneficial for avoiding normal tissue damage, such as in hepatitis (43, 44). In contrast, it has been shown that TRAIL exerts no apparent toxicity in vivo (44). Therefore, the specific upregulation of TRAIL by type I IFNs can be expected to augment T cell cytotoxicity against tumor cells without harmful effects on normal tissues.

Although this study demonstrated a potential role of TRAIL in mediating T cell cytotoxicity against tumor cells, physiological and pathological roles of TRAIL-mediated T cell cytotoxicity remain largely unknown. It is well known that type I IFNs can be inducible by infection of various viruses, bacteria, and mycoplasmas (21, 22). In this respect, TRAIL-mediated T cell cytotoxicity may play an important role in clearance of virus- or intracellular organism–infected cells, which has been shown to be dependent on type I IFNs but not on perforin or FasL (14, 45). It is also interesting to note that TRAIL might be involved in the pathogenesis of AIDS. Recently, Jeremias et al. reported that PBT cells from HIV-infected patients but not those from healthy individuals were susceptible to TRAIL-induced apoptosis (34). Since elevated serum levels of IFN-α were found during HIV infection (46), it is possible that type I IFN-induced, TRAIL-dependent T cell cytotoxicity might be involved in clearance of HIV-infected cells or in the pathogenesis of AIDS and HIV-associated diseases. This notion is further supported by a recent report showing a contribution of TRAIL to anti-CD3–induced apoptosis of PBT cells from HIV-infected patients (47). Further studies are now underway to address these issues.

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References

1. Wiley, S.R., K. Schooley, P.J. Smolak, W.S. Din, C.P. Huang, J.K. Nicholl, G.R. Sutherland, T.D. Smith, C. Rauch, C.A. Smith, and R.G. Goodwin. 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*. 3:673–682.
2. Pitti, R.M., S.A. Marsters, S. Ruppert, C.J. Donahue, A. Moore, and A. Ashkenazi. 1996. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J. Biol. Chem*. 271:12687–12690.
3. Thomas, W.D., and P. Hersey. 1998. TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in Fas ligand-resistant melanoma cells and mediates CD4 T cell killing of target cells. *J. Immunol*. 161:2195–2200.
4. Nagata, S. 1997. Apoptosis by death factor. *Cell*. 88:355–365.
5. Pan, G., K. O'Rourke, A.M. Chinnaiyan, R. Gentz, R. Ebner, J. Ni, and V.M. Dixit. 1997. The receptor for the cytotoxic ligand TRAIL. *Science*. 276:111–113.
6. Sheridan, J.P., S.A. Marsters, R.M. Pitti, A. Gurney, M. Skubatch, D. Baldwin, L. Ramakrishnan, C.L. Gray, K. Baker, W.I. Wood, et al. 1997. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science*. 277:818–821.
7. Sheikh, M.S., T.F. Burns, Y. Huang, G.S. Wu, S. Amundson, K.S. Brooks, A.J. Fornace, Jr., and W.S. el-Deiry. 1998. p53-dependent and -independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor α. *Cancer Res*. 58:1593–1598.
8. Pan, G., J. Ni, Y.F. Wei, G. Yu, R. Gentz, and V.M. Dixit. 1997. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science*. 277:815–818.
9. Degli-Esposti, M.A., W.C. Dougall, P.J. Smolak, J.Y. Waugh, C.A. Smith, and R.G. Goodwin. 1997. The novel receptor TRAIL-R4 induces NF-κB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity*. 7:813–820.
10. Ashkenazi, A., and V.M. Dixit. 1998. Death receptors: signaling and modulation. *Science*. 281:1305–1308.
11. Kägi, D., F. Vignaux, B. Ledermann, K. Burki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science*. 265:528–530.
12. Kojima, H., N. Shinohara, S. Hanaoka, Y. Someya-Shirota, Y. Takagaki, H. Ohno, T. Saito, T. Katayama, H. Yagita, K. Okumura, et al. 1994. Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. *Immunity*. 1:357–364.
13. Lowin, B., M. Hahne, C. Mattmann, and J. Tschopp. 1994. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature*. 370:650–652.
14. Kägi, D., B. Ledermann, K. Burki, R.M. Zinkernagel, and H. Hengartner. 1996. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. *Annu. Rev. Immunol*. 14: 207–232.
15. van den Broek, M.E., D. Kägi, F. Ossendorp, R. Toes, S. Vamvakas, W.K. Lutz, C.J. Melief, R.M. Zinkernagel, and H. Hengartner. 1996. Decreased tumor surveillance in perforin-deficient mice. *J. Exp. Med*. 184:1781–1790.
16. Davidson, W.F., T. Giese, and T.N. Fredrickson. 1998. Spontaneous development of plasmacytoid tumors in mice with defective Fas–Fas ligand interactions. *J. Exp. Med*. 187: 1825–1838.
17. Braun, M.Y., B. Lowin, L. French, H. Acha-Orbea, and J. Tschopp. 1996. Cytotoxic T cells deficient in both functional fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal acute graft-versus-host disease. *J. Exp. Med*. 183:657–661.
18. Hattori, K., T. Hirano, H. Miyajima, N. Yamakawa, M. Tateno, K. Oshimi, N. Kayagaki, H. Yagita, and K. Okumura. 1998. Differential effects of anti-Fas ligand and anti-tumor necrosis factor α antibodies on acute graft-versus-host disease pathologies. *Blood*. 91:4051–4055.
19. Seino, K., N. Kayagaki, K. Takeda, K. Fukao, K. Okumura, and H. Yagita. 1997. Contribution of Fas ligand to T cell-mediated hepatic injury in mice. *Gastroenterology*. 113:1315–1322.
20. Kayagaki, N., N. Yamaguchi, M. Nakayama, A. Kawasaki, H. Akiba, K. Okumura, and H. Yagita. 1999. Involvement of TNF-related apoptosis-inducing ligand in human $CD4^+$ T cell-mediated cytotoxicity. *J. Immunol*. 162:2639–2647.
21. Morris, A., and I. Zvetkova. 1997. Cytokine research: the interferon paradigm. *J. Clin. Pathol*. 50:635–639.
22. Gresser, I. 1997. Wherefore interferon? *J. Leukocyte Biol*. 61: 567–574.
23. Strander, H. 1986. Interferon treatment of human neoplasia. *Adv. Cancer Res*. 46:1–265.
24. Gutterman, J.U. 1994. Cytokine therapeutics: lessons from interferon α. *Proc. Natl. Acad. Sci. USA*. 91:1198–1205.
25. Okada, Y., K. Gohji, S. Kamidono, and O. Tachiwaki. 1986. Establishment and characterization of a cell line (KO-RCC-1) from a human renal cell carcinoma. *Jpn. J. Urol*. 77:780–785.
26. Kataoka, T., N. Shinohara, H. Takayama, K. Takaku, S. Kondo, S. Yonehara, and K. Nagai. 1996. Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. *J. Immunol*. 156:3678–3686.
27. Kayagaki, N., A. Kawasaki, T. Ebata, H. Ohmoto, S. Ikeda, S. Inoue, K. Yoshino, K. Okumura, and H. Yagita. 1995. Metalloproteinase-mediated release of human Fas ligand. *J. Exp. Med*. 182:1777–1783.
28. Miyatake, S., H. Nakano, S.Y. Park, T. Yamazaki, K. Takase, H. Matsushime, A. Kato, and T. Saito. 1995. Induction of G1 arrest by down-regulation of cyclin D3 in T cell hybridomas. *J. Exp. Med*. 182:401–408.
29. Nakata, M., A. Kawasaki, M. Azuma, K. Tsuji, H. Matsuda, Y. Shinkai, H. Yagita, and K. Okumura. 1992. Expression of perforin and cytolytic potential of human peripheral blood lymphocyte subpopulations. *Int. Immunol*. 4:1049–1054.
30. de Boer, M., A. Kasran, J. Kwekkeboom, H. Walter, P. Van-

denberghe, and J.L. Ceuppens. 1993. Ligation of B7 with CD28/CTLA-4 on T cells results in CD40 ligand expression, interleukin-4 secretion and efficient help for antibody production by B cells. *Eur. J. Immunol.* 23:3120–3125.

31. Ye, W., J.D. Young, and C.C. Liu. 1996. Interleukin-15 induces the expression of mRNAs of cytolytic mediators and augments cytotoxic activities in primary murine lymphocytes. *Cell. Immunol.* 174:54–62.
32. Tsutsui, T., J. Mu, M. Ogawa, W.G. Yu, T. Suda, S. Nagaga, F. Saji, Y. Murata, H. Fujiwara, and T. Hamaoka. 1997. Administration of IL-12 induces a $CD3^+$ $CD4^-$ $CD8^-$ $B220^+$ lymphoid population capable of eliciting cytolysis against Fas-positive tumor cells. *J. Immunol.* 159:2599–2605.
33. Tsutsui, H., K. Nakanishi, K. Matsui, K. Higashino, H. Okamura, Y. Miyazawa, and K. Kaneda. 1996. IFN-γ-inducing factor up-regulates Fas ligand-mediated cytotoxic activity of murine natural killer cell clones. *J. Immunol.* 157:3967–3973.
34. Jeremias, I., I. Herr, T. Boehler, and K.M. Debatin. 1998. TRAIL/Apo-2-ligand-induced apoptosis in human T cells. *Eur. J. Immunol.* 28:143–152.
35. Darnell, J.E., Jr., I.M. Kerr, and G.R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science.* 264:1415–1421.
36. Kaido, T.J., C. Maury, and I. Gresser. 1995. Host $CD4^+$ T lymphocytes are required for the synergistic action of interferon-α/β and adoptively transferred immune cells in the inhibition of visceral ESb metastases. *Cancer Res.* 55:6133–6139.
37. Ferrantini, M., M. Giovarelli, A. Modesti, P. Musiani, A. Modica, M. Venditti, E. Peretti, P.L. Lollini, P. Nanni, G. Forni, and F. Belardelli. 1994. IFN-α 1 gene expression into a metastatic murine adenocarcinoma (TS/A) results in $CD8^+$ T cell-mediated tumor rejection and development of antitumor immunity. Comparative studies with IFN-γ-producing TS/A cells. *J. Immunol.* 153:4604–4615.
38. Kaido, T., I. Gresser, C. Maury, M.T. Maunoury, F. Vignaux, and F. Belardelli. 1993. Sensitized T lymphocytes render DBA/2 beige mice responsive to IFN α/β therapy of Friend erythroleukemia visceral metastases. *Int. J. Cancer.* 54: 475–481.
39. Kaido, T., C. Maury, V. Schirrmacher, and I. Gresser. 1994. Successful immunotherapy of the highly metastatic murine ESb lymphoma with sensitized $CD8^+$ T cells and IFN-α/β. *Int. J. Cancer.* 57:538–543.
40. Romero, P., P. Rod Dunbar, D. Valmori, M. Pittet, G.S. Ogg, D. Rimoldi, J.L. Chen, D. Lienard, J.C. Cerottini, and V. Cerundolo. 1998. Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes. *J. Exp. Med.* 188:1641–1650.
41. Jantzer, P., and D.J. Schendel. 1998. Human renal cell carcinoma antigen-specific CTLs: antigen-driven selection and long-term persistence in vivo. *Cancer Res.* 58:3078–3086.
42. Yagita, H., N. Kayagaki, A. Kawasaki, and K. Okumura. 1997. CD95 ligand pre-workshop report. *In* Leukocyte Typing VI. Garland Publishing, Inc., New York. 105–109.
43. Tanaka, M., T. Itai, M. Adachi, and S. Nagata. 1998. Down-regulation of Fas ligand by shedding. *Nat. Med.* 4:31–36.
44. Schneider, P., N. Holler, J.L. Bodmer, M. Hahne, K. Frei, A. Fontana, and J. Tschopp. 1998. Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. *J. Exp. Med.* 187:1205–1213.
45. van den Broek, M.F., U. Muller, S. Huang, R.M. Zinkernagel, and M. Aguet. 1995. Immune defence in mice lacking type I and/or type II interferon receptors. *Immunol. Rev.* 148: 5–18.
46. Ambrus, J.L., B.J. Poiesz, M.A. Lillie, I. Stadler, L.A. Di Berardino, and K.C. Chadha. 1989. Interferon and interferon inhibitor levels in patients infected with varicella-zoster virus, acquired immunodeficiency syndrome, acquired immunodeficiency syndrome-related complex, or Kaposi's sarcoma, and in normal individuals. *Am. J. Med.* 87:405–407.
47. Katsikis, P.D., M.E. Garcia-Ojeda, J.F. Torres-Roca, I.M. Tijoe, C.A. Smith, and L.A. Herzenberg. 1997. Interleukin-1 β converting enzyme-like protease involvement in Fas-induced and activation-induced peripheral blood T cell apoptosis in HIV infection. TNF-related apoptosis-inducing ligand can mediate activation-induced T cell death in HIV infection. *J. Exp. Med.* 186:1365–1372.