Signaling through the Lymphotoxin β Receptor Induces the Death of Some Adenocarcinoma Tumor Lines

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Summary

Surface lymphotoxin (LT) is a heteromeric complex of LT-α and LT-β chains that binds to the LT-β receptor (LT-β-R), a member of the tumor necrosis factor (TNF) family of receptors. The biological function of this receptor-ligand system is poorly characterized. Since signaling through other members of this receptor family can induce cell death, e.g., the TNF and Fas receptors, it is important to determine if similar signaling events can be communicated via the LT-β-R. A soluble form of the surface complex was produced by coexpression of LT-α and a converted form of LT-β wherein the normally type II LT-β membrane protein was changed to a type I secreted form. Recombinant LT-α/β₂ was cytotoxic to the human adenocarcinoma cell lines HT-29, WiDr, MDA-MB-468, and HT-3 when added with the synergizing agent interferon (IFN) γ. When immobilized on a plastic surface, anti-LT-β-R monoclonal antibodies (mAbs) induced the death of these cells, demonstrating direct signaling via the LT-β-R. Anti-LT-β-R mAbs were also identified that inhibited ligand-induced cell death, whereas others were found to potentiate the activity of the ligand when added in solution. The human WiDr adenocarcinoma line forms solid tumors in immunocompromised mice, and treatment with an anti-LT-β-R antibody combined with human IFN-γ arrested tumor growth. The delineation of a biological signaling event mediated by the LT-β-R opens a window for further studies on its immunological role, and furthermore, activation of the LT-β-R may have an application in tumor therapy.

The TNF family of ligands and receptors is a set of regulatory elements in the immune system (1). TNF was discovered as a cytolytic agent circulating in the blood of endotoxin-stimulated animals (2-4). Originally cloned in the expectation that TNF would be a novel antitumor agent, it was later shown that its primary physiologic function lies in initiating the inflammatory cascade underlying the host’s immediate defensive response to infection or stress. More complex immunological functions have been described (5, 6). Lymphotoxin (LT)α (also called TNF-β) is a similar cytokine secreted by activated lymphocytes (7) and was originally characterized as having the same functions as TNF. Later, activated T and B cells were found to display LT-α on their surfaces in an unusual form complexed with another member of the TNF family called LT-β in an LT-α₁/β₂ stoichiometry (8-13). A complex with an apparent LT-α₂/β₁ stoichiometry is also present, but only in minor amounts on human lymphocytes. The major LT-α₁/β₂ form does not bind to the known TNF receptors, referred to here as TNF-R55 and TNF-R75, but rather interacts with another receptor in the TNF family called the LT-β receptor (LT-β-R) (9, 14).

Currently, the function of the LT system is poorly characterized, however, there are suggestions that LT signaling is involved in the development of the peripheral lymphoid organs. Genetic disruption of the LT-α gene in mice led to an unusual phenotype. The mice lacked lymph nodes and lost the organization of T and B cells in the follicles in the spleen (15, 16). A similar loss of lymph nodes occurs in dly, mice although this mouse, unlike the LT-α knockout mouse, is severely immunocompromised (17). Signaling through the two known TNF receptors has not been shown to mediate the development of the lymph nodes since knockout of either receptor does not lead to the loss of lymph nodes (18, 19). Thus, it has been postulated that signaling through the LT-β-R constitutes a regulatory pathway that is distinct from TNF-related events and may...
Activation of several members of the TNF family of receptors can have cytotoxic or growth-inhibitory consequences (1). For example, Fas receptor activation results in apoptosis of many cell types, including both transformed and non-transformed cells (20, 21), and this process is likely to play a role in the deletion of autoreactive lymphocytes in the periphery (22). TNF and LT-α also can kill some transformed cells, and it is likely that tumor cells respond abnormally by either necrosing or apoptosing to what is normally a differentiation-like signal. More recently, TNF signaling has been proposed to induce the death of nontransformed lymphoblasts in a slow fashion (23, 24), and this process appears to require TNF-R75. The physiological significance of this event remains to be explored. The Fas receptor and the TNF-R55 both possess a unique cytoplasmic domain, called the death domain, that is required to initiate cell death (25). CD30 and CD40 signaling can inhibit growth and may also induce apoptosis, yet these receptors as well as the TNF-R75 and the LT-β-R lack obvious death domains (12, 26, 27). We have investigated whether LT-β-R signaling could induce cell death both because of its possible immunological relevance and to provide a practical starting point for studying the role of the LT system. Using either recombinant ligands or antireceptor mAbs with agonist activity, the ability of LT-β-R activation to induce cell death in various transformed lines was examined. In this report, we show that LT-β-R signaling can induce cell death in a limited group of adenocarcinoma tumor lines.

Materials and Methods

Cells. All cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD) except for WEHI 164 clone 13, which was obtained from Dr. Eric Kawashima (Glaxo Institute for Molecular Biology, Geneva, Switzerland). WEHI 164 cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS). HT-29 and WiDr cells were maintained in MEM with Earle’s salts, 10% FCS with glutamine, penicillin/streptomycin, nonessential amino acids, and sodium pyruvate. These two cell lines are heterogenous in their response to the presence of 50 U/ml IFN-γ with various dilutions of TNF, anti-LT-α-R mAbs, and LT-α3 μg/ml 

Recombinant Cytokines. Human TNF and IFN-γ were produced at Biogen (30). Recombinant human LT-α was prepared by expression in insect cells as described (31) and was similar to material expressed in CHO cells (30). The recombinant LT-α/β heteromorphic forms were prepared by coinfection of insect cells with two baculoviruses encoding the human LT-α and human LT-β proteins (Browning, J.L., K. Miatkowski, D.A. Griffiths, P.R. Bourdon, C. Hession, C.M. Ambrose, and W. Meier, manuscript in preparation). The transmembrane region of the LT-β gene was replaced with a vascular cell adhesion molecule (VCAM) leader sequence to enable secretion of mixed LT-α/β forms. The trimers LT-α1/β2, LT-α2/β1, and LT-α3 were purified using combinations of p55 TNF-R and LT-β-R affinity columns. The resultant preparations have been well characterized, contain only LT forms, are trimeric, and are >95% pure with respect to LT forms based on ion exchange chromatographic resolution of the three stoichiometrically different trimers. The LT-α1/β2 preparation contained <1 part in 5,000 of LT-α-like activity as assessed using the WEHI 164 indicator line and by comparison of various LT trimers prepared with a LT-α D50N mutation that eliminates TNF-R binding (Browning J.L., K. Miatkowski, D.A. Griffiths, P.R. Bourdon, C. Hession, C.M. Ambrose, and W. Meier, manuscript in preparation).

Cytotoxicity Assays. In the cytotoxicity assays, serial dilutions of the cytokines or antibodies were prepared in 50 μl of 96-well plates, and 5,000 HT-29-14 cells were added in 50 μl of media with or without IFN-γ. After 3–4 d, 10 μl of 5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide] was added, and after 3 h the formazan was dissolved by adding 100 μl of 10% SDS in 10 mM HCl. After a further 24-h incubation at 37°C, the OD was quantitated at 550 nm. In some experiments, soluble receptor forms or pure human IgG were added in 10 μl before the addition of the cells. To immobilize mAbs on the plastic surface, 96-well tissue culture plates were first coated with 50 μl of 10 μg/ml affinity-purified goat anti-mouse Fc polyclonal antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), washed with 5% FBS in PBS, and then coated with varying amounts of the various mAbs diluted into tissue culture media with FBS. The plates were washed with media before use. Cells were added and growth assessed as indicated above. To survey the panel of cells shown in Table 4, cells were plated in the presence of 50 U/ml IFN-γ with various dilutions of TNF, anti-Fas, LT-α/β, or into wells coated with various anti-LT-β-R mAbs as described above. Growth was assessed with the MTT readout, and wells were also visually inspected for morphology changes. Dramatic growth inhibition was scored as two pluses (+ +), partial growth inhibition at reasonable concentrations was noted as one plus (+), and partial effects requiring high concentrations of ligand was marked as plus/minus (+/−).

Mouse Anti-LT-β-R mAbs. Mouse hybridomas producing mAbs to the human LT-β-R were prepared by immunization of RBF mice with the LT-β-R–Fc chimera essentially as described previously (9). All mAbs were IgG1 isotypes.

FACS® Binding Assays. To monitor receptor binding to surface ligand, 200 ng/ml soluble LT-β-R–Fc were added to PMA-activated II-23 T cell hybridoma cells, and binding was detected using a PE-labeled donkey anti-mouse Ig essentially as described previously (9). To assess the blocking ability of the mAbs, mAbs were preincubated with the soluble receptor for 20 min before addition of the receptor-mAb mixture to the II-23 cells. In other experiments, receptor expression on adherent tumor lines was determined by FACS® analysis of cells removed with PBS with 5 mM EDTA and stained using anti-LT-β-R mAbs and a PE-labeled
donkey anti-mouse IgG reagent (Jackson ImmunoResearch Laboratories).

**Epitope Mapping by BLAcore™ Analysis.** Affinity-purified goat anti-human Fc (Jackson ImmunoResearch Laboratories) was immobilized onto an N-hydroxysuccinimide-activated sensor chip, and LT-β-R-Fc was captured onto the anti-human-Fc-coated chip. The various pairs of anti-LT-α/β mAbs were then bound sequentially, and the ability of the second mAb to bind in the presence of the first mAb was measured using a BLAcore™ 2000 (Pharmacia Biosensor, Uppsala, Sweden). The entire array of 49 mAb combinations were assessed for cross-blocking in this manner and analyzed essentially as described (32).

**Analysis of Apoptosis.** Terminal deoxynucleotidyl transferase UTP nick-end labeling (TUNEL) of free DNA ends, i.e., TUNEL staining, was carried out using the ApopTag™ kit (Oncor Inc., Gaithersburg, MD) according to the manufacturer's specifications.

**Tumor Growth in SCID Mice.** BALB/c SCID female mice at 6–8 wk old (The Jackson Laboratory, Bar Harbor, ME) were injected with 10⁶ trypsinized and washed WiDr cells in a volume of 0.2 ml subcutaneously onto the back of the animal. Mice were treated with or without antibody either with or without 10⁶ antiviral U/mouse of human IFN-γ by intraperitoneal injection in 0.2 ml on days 0 and 1 or as indicated. The amounts of IFN-γ and antibody have not been optimized. Tumor volume was calculated from the radius as determined by caliper measurements in two dimensions. The results shown in Fig. 6 B were determined in a blinded format.

**Results**

**LT-α/β2 Is Cytotoxic to HT-29 Cells.** Recombinant LT-α/β2 and LT-α/β1 trimers were tested for their ability to inhibit the growth of a number of tumor lines. IFN-γ was included in the screening as it had been shown to enhance the cytolytic properties of TNF (33). Recombinant soluble LT-α/β2 inhibited the growth of human HT-29 cells only in the presence of IFN-γ (Fig. 1) and, as shown previously, this cell line was also sensitive to the anti-Fas receptor mAb CH11, TNF, and LT-α (30, 34, 35). IFN-α and -β were 100-fold less effective when compared on the basis of antiviral units (data not shown). Soluble LT-α/β1 was much less active in this assay.

The specificity of the LT-α/β2 cytotoxicity was examined in several ways. Soluble TNF-R55 and LT-β-R immunoglobulin chimeras (TNF-R55–Fc and LT-β-R–Fc) were tested for their ability to block the various activities (9). These receptors can bind to the appropriate cleft between two subunits in the trimeric ligand structures and interfere with the ability of receptor on the membrane to bind ligand. As expected and shown previously (36, 37), TNF-R55–Fc completely blocked TNF-induced growth inhibition by binding to TNF and preventing its interaction with surface receptor (Table 1). Soluble TNF-R had no effect on LT-α/β2-mediated antiproliferative effects. In contrast, LT-β-R–Fc blocked LT-α/β2 effects, but not those of TNF or LT-α. Moreover, anti-LT-α–neutralizing mAbs (9) did not affect the LT-α/β2 cytotoxicity (data not shown), confirming that soluble trace LT-α contaminants were not involved in the activity of LT-α/β2 on HT-29 cells. Additionally, a mutated form of LT-α (D50N) that lacks the ability to signal through the TNF-R was examined (38). LT-α/β2 prepared with the mutant LT-α retained essentially full activity on HT-29 and WiDr cells (see below), further eliminating TNF-R55 binding as a possible mechanism for the cytotoxic effects of LT-α/β2 (Ambrose, C., unpublished data). An anti-TNF neutralizing mAb 104c, also had no effect on LT-α/β2 activity, precluding the induction of TNF synthesis as a mechanism for the LT-α/β2 effects. These assays indicate that LT-α/β2 can trigger cytotoxic events via non–TNF-R–mediated mechanisms.

**Mechanism of Growth Inhibition.** The growth inhibition assay alone does not discriminate between death and stasis; however, direct observation of the treated HT-29 cells showed that Fas receptor activation led to rapid cell death, i.e., within 12–24 h, whereas TNF effects were slower and required ~24 h. LT-α/β2-treated cells underwent a much slower death, with dead cells not being visible until 1.5–2 d. The morphology of the dying cells was identical in all three cases. Some cells have the appearance of apoptotic bodies with condensed nuclei, whereas in others the nucleus appears to condense and the cytoplasm balloons out and becomes clear (Fig. 2). Substantial cell lysis does not occur even after 3–4 d. The TNF-, anti-Fas receptor mAb– or LT-α/β2-treated populations had more cells that stained brightly with HOECHST dye staining (data not shown), which can be indicative of chromatin condensation accompanying apoptotic events. Internucleosomal DNA fragmen-
Within each group, the mAbs effectively blocked each ble LT-\(\beta\)-R-Fc binding to surface ligand on PMA-activated cells. These mAbs were grouped into four subsets based on epitope, and a panel of mouse anti-LT-\(\alpha\)-P\(_{55}\) mAbs were isolated and characterized similar LT-\(\alpha\)-R-specific mAbs.

Hydroxyl ends of DNA, which is more sensitive than DNA after all three treatments. This large fragment is reminiscent of the large 50–200-kb cleavage products previously described in dying epithelial tumor cells (39, 40). Some DNA fragmentation was observed using TUNEL staining of 3’ hydroxyl ends of DNA, which is more sensitive than DNA laddering (Fig. 3). Therefore, the death induced by TNF-\(\beta\)-R, Fas, or LT-\(\beta\)-R signaling may be basically apoptotic even though all of the classic features of apoptosis have not been observed in epithelial tumors (39, 41).

Properties of Anti-LT-\(\beta\)-R mAbs. Antibodies to receptors in the TNF family can have either antagonistic or agonistic effects, and as tools they have been very useful in delineating the consequences of receptor activation. To determine if LT-\(\beta\)-R cross-linking could induce cell death, we prepared and characterized similar LT-\(\beta\)-R–specific mAbs. Mice were immunized with the LT-\(\beta\)-R–Fc fusion protein, and a panel of mouse anti-LT-\(\beta\)-R mAbs were isolated. These mAbs were grouped into four subsets based on their performance in the following assays: (a) the ability to cross-block each other in a mAb/antigen-binding experiment using plasmon resonance detection (i.e., a BIAcore instrument defined epitope mapping experiment); (b) the ability to block soluble LT-\(\beta\)-R–Fc binding to surface ligand on PMA-activated II-23 cells; and (c) the ability to affect LT-\(\alpha\)/\(\beta\)–induced cell death. The results of this analysis are summarized in Table 2.

Epitope mapping using the BIAcore instrument defined four separate epitopes recognized by this panel of mAbs and formed the basis of the grouping shown in Table 2. Within each group, the mAbs effectively blocked each other. The group I mAbs did not cross-block any other mAbs. The group II epitope partially overlapped the group III epitope. We identified only one mAb, CBE11, with group IV properties, and its epitope slightly overlapped the BCG6 and BKA11 sites. To directly assess the ability of the mAbs to inhibit receptor–ligand binding, a FACS® binding assay was used. Antibodies were premixed with soluble receptor, and the ability of the receptor to bind to ligand on the surface of II-23 cells was quantitated. This assay has the advantage that any agonistic activity of the antireceptor mAbs would not be apparent. mAbs from groups I, II, and IV were effective inhibitors, suggesting that they bind close to the ligand binding region of the receptor (Table 2).

In the HT-29 cytotoxicity assay, all mAbs in these groups either weakly inhibited growth or lacked activity when simply added to the culture in the absence of ligand (Fig. 4A). There was considerable variability in this assay possibly reflecting differing receptor densities at the start of the experiment; e.g., compare Fig. 4A and Table 3. In dramatic contrast, when the mAbs were immobilized on the plastic, all mAbs were found to have potent cytotoxic activity (Fig. 4B), and this activity was again completely dependent on the presence of IFN-\(\gamma\). This result demonstrates that LT-\(\beta\)-R cross-linking is sufficient to induce cell death. Moreover, we reasoned that mAbs to two different epitopes should be able to cross-link receptors more effectively, and indeed Table 3 shows that certain pairs of mAbs were cytotoxic in solution. In general, mAbs from two different groups had to be paired to get cytotoxic activity.

In addition to the direct agonistic effects of the mAbs described above, we investigated the effects of the mAbs on ligand induced cell death. Each of the four groups of mAbs had differing effects reinforcing the grouping based on BIAcore epitope mapping. Group I mAbs primarily blocked LT-\(\alpha\)/\(\beta\) activity with a small amount of direct growth inhibition occurring in the absence of ligand (Fig. 4C). In some experiments (data not shown), a Fab fragment of BDA8 was used, and the small growth inhibitory activity disappeared and only direct inhibition was observed. Such complex mixed agonist/antagonist effects have been observed with anti-CD40 mAbs (42). The group II mAbs had complex effects that suggested mixed agonistic and antagonistic behavior (data not shown). Group III mAbs potenti-ated LT-\(\alpha\)/\(\beta\) activity presumably by creating local regions of high receptor density that would enhance ligand mediated cross-linking (Fig. 4D), and there was no evidence of antagonistic effects. The cytotoxic effects of TNF were also slightly potentiated by these mAbs, suggesting that certain signal transduction elements were facilitating or priming the cells for TNF signaling. The group IV mAb, CBE11, did not affect ligand-mediated cytotoxicity. Because the LT-\(\beta\)-R–specific mAbs can directly effect death, one can conclude that the LT-\(\beta\)-R is able to signal cell death, and this event accounts for the cytotoxic activity of LT-\(\alpha\)/\(\beta\).

Survey of Sensitive Cell Lines. A series of human tumor lines have been screened for sensitivity to cytotoxic or growth-inhibitory effects of either the LT-\(\alpha\)/\(\beta\) ligand or plastic immobilized anti-LT-\(\beta\)-R mAbs in the presence of IFN-\(\gamma\) (summarized in Table 4). The WiDr line (43) was found to be sensitive to LT-\(\alpha\)/\(\beta\) in a very similar manner as the HT-29 line, and it is likely that this line is actually a
Figure 2. Photograph of HT-29 cells treated with IFN-γ (80 U/ml) alone or IFN-γ plus 50 ng/ml LTα1/β2 for 3 d ×200.

There was no evidence that LT-β-R signaling was cytostatic to normal human fibroblasts, endothelial cells, or primary lymphocytes, which was expected since lymphocytes do not express the receptor (Hochman, P., J.L. Browning, and C. Ware, unpublished observations). The growth of the human diploid fibroblastoid line WI-38 was stimulated by LT-β-R signaling (data not shown). The adenocarcinoma lines SW620 and SW1417 both displayed altered morphology in response to LT-β-R signaling without growth arrest.

*Anti*-LT-β-R mAb Inhibits the Growth of WiDr in SCID Mice. We have explored the ability of an anti–LT-β-R mAb, CBE11, to block the growth of the WiDr line in immunodeficient mice. When the mice were treated intraperitoneally with the CBE11 mAb at the same time as the WiDr cells were inoculated subcutaneously, tumor outgrowth was dramatically blocked (Fig. 6 A). The antitumor action was enhanced by IFN-γ; however, the mAb was effective even without exogenous IFN-γ. In the CBE11 + IFN-γ group, 7 of 16 animals completely lacked tumors, whereas the remaining animals had small nodules that had not progressed at 2 mo. The CBE11 animals treated without IFN-γ were similar to the CBE11 + IFN-γ group at 30 d; however, these mice eventually developed slowly growing tumors. There were statistically significant differences between the control or IFN-γ-treated groups and the CBE11-treated groups, whereas no significant differences were observed between the saline, IFN-γ, or the control anti-human LFA3 mAb (1E6) + IFN-γ groups. The 1E6 and CBE11 mAbs are both IgG1s, and since the 1E6 mAb effectively coats the tumor line yet did not block tumor

derivative of HT-29 (28). The breast adenocarcinoma MDA-MB-468 and cervical carcinoma HT-3 lines were growth inhibited, and whether cell death occurred was not rigorously determined. In general, however, the majority of tumor lines are not sensitive under these relatively fixed conditions. This survey was complicated by the fact that the sensitivity of the cells to IFN-γ varied dramatically, and the lines are often heterogenous, with mixtures of responsive and nonresponsive cells obfuscating a simple MTT-based test.

Cell surface levels of LT-β-R were examined to determine whether LT-β-R presence is the determining factor for sensitivity to the LT ligand. Fig. 5 shows a comparison of LT-β-R and CD40 receptor levels on four different tumor lines. The CD40 staining is included since its surface levels are very abundant on some epithelial tumor lines, as is the LT-β-R. All four cell lines are LT-β-R positive, and these results are typical of most nonlymphoid cells (Mackay, F., and J.L. Browning, unpublished results). Despite the presence of LT-β-R on all four cell types, only HT-29 cells are appreciably sensitive to LT-α1/β2, indicating that sensitivity does not correlate simply with receptor presence. In the TNF, Fas, and CD40 signaling systems, no correlation has ever been observed between the level of surface receptor and whether a cell type responds biologically. Relative to Fas, TNF-R55, and TNF-R75, LT-β-R is very abundant on cells. Experiments on the HT-29 and WiDr lines indicated that IFN-γ treatment did not upregulate the LT-β-R, whereas in the same experiments the Fas receptor was dramatically upregulated as described previously (35).
growth, we can exclude complement- or NK cell-mediated events as the basis for the tumor inhibition. The efficacy of CBE11 in the absence of IFN-γ was unexpected since there was an absolute dependence on IFN-γ for any LT-β-R based in vitro cytotoxic effect. Either there is some crossover of mouse IFN-γ onto human IFN-γ receptors, or other mechanisms are involved in vivo. The mechanism(s) by which LT-β-R signaling prevents tumor growth in vivo effects are being investigated. The ability of combined IFN-γ/CBE11 treatment to inhibit the growth of an established tumor was demonstrated (Fig. 6 B). Mice were inoculated with 10⁶ WiDr cells, and after 15 d, treatment was initiated. At this point the average tumor volume was 0.076 cc or an average diameter of 0.532 cm. Treated tumors stopped growing, and after three injections of antibody over 3 wk, growth was arrested out to 7 wk after inoculation, at which point the experiment was terminated.

**Discussion**

Activation of the receptors of the TNF family can direct cells into a proliferative or differentiation type response, or it can induce cell death sometimes even in the same cell type depending on the conditions (4, 22, 44). In this work, we have shown that signaling through the LT-β-R leads to the death of the HT-29 and WiDr human adenocarcinoma cell lines and is at least growth inhibitory to two other lines. This activity represents the first observed effect of LT-β-R signaling and is important not only because of the current interest in cytotoxic events, but because it provides a biological assay for characterizing various reagents. There was no evidence that LT-β-R signaling was cytostatic to

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**Table 2. Summary of Mouse Anti-Human LT-β-R mAbs**

| mAb group | mAb name | Cell staining | Blocking receptor binding | mAb immobilized on Plastic | Soluble mAb alone | Soluble mAb with LT-α,β |
|-----------|----------|---------------|---------------------------|-----------------------------|-------------------|------------------------|
| I         | BDA8     | +++           | +++                       | +                           | +/+               | Inhibits               |
| I         | AGH1     | +++           | +++                       | +                           | +/+               | Inhibits               |
| II        | BCG6     | +++           | +                         | +                           | +/-               | Mixed                  |
| II        | BHA10    | +++           | +                         | +                           | +/-               | Mixed                  |
| III       | BKA11    | +++           | +/-                       | +                           | +/-               | Potentiates            |
| III       | CDH10    | +++           | +/-                       | +                           | +/-               | Potentiates            |
| IV        | CBE11    | +++           | +++                       | +                           | +/-               | No effect              |
| Controls  | MOPC21   | -             | -                         | -                           | -                 | No effect              |
|           | HT29/26  | -             | ND                        | -                           | -                 | No effect              |
|           | TS 2/94  | ND            | ND                        | -                           | -                 | No effect              |

*FACS staining of CHO cells transfected with LT-β-R.
†Assay assessed whether antibody blocks binding of soluble receptor to the activated T cell hybridoma II-23.
‡HT-29 cells were grown with IFN-γ on anti-LT-β-R–coated plates as described in Materials and Methods.
§Variable, partial inhibition in some assays, no effects in others.
¶Anti–human LFA-3, a mouse IgG1.
Table 3. Effect of Pairs of Anti-LT-β-R mAbs on HT-29/WiDr Growth

| mAb Concentration (ng/ml) | mAb Concentration (ng/ml) | Cell growth (OD 550 nm) |
|---------------------------|---------------------------|------------------------|
| IgG1 200                  | BDA8 100                  | 0.77 (HT-29)           |
| —                        | BCG6 100                  | 0.50                   |
| CDH10 33                  | AGH1 50                   | 0.49                   |
| CDH10 33                  | CBE11 50                  | 0.23                   |
| IgG1 100                  | CDH10 10                  | 0.80 (HT29)            |
| CDH10 10                  | CBE11 50                  | 0.86                   |
| IgG1 50                   | CDH10 10                  | 0.81                   |
| —                        | CBE11 50                  | 0.58                   |
| CDH10 10                  | AGH1 50                   | 0.21                   |
| BHA10 10                  | AGH1 50                   | 0.36                   |
| CDH10 10                  | AGH1 50                   | 0.16                   |

Dotted lines indicate separate experiments.

*Typically, further increases in the mAb concentration did not increase the amount of growth inhibition.

nontransformed cells, and signaling could actually drive growth in some situations. This pattern of cell sensitivity to LT-α/β₂ basically resembles the effects of TNF. The data we have obtained suggest that the cytotoxicity mediated by the LT-β-R will be limited to transformed cells. Recently, signaling through TNF-R75 has been reported to mediate an unusually slow death of nontransformed activated lymphocytes (23, 24), indicating that TNF-induced death may not be limited to transformed cells. In contrast, nontransformed lymphocytes can be induced to undergo rapid apoptosis after Fas signaling, and this event is most likely to be involved in the deletion of certain lymphocyte populations in the periphery (22, 45). Whether the slow events involving LT-β-R or TNF-R75 are physiologically important will require further investigation. Surface LT is abundant on lymphokine activated T cells, i.e., LAK cells (12, 46). Currently, cell mediated cytotoxicity is thought to be mediated through the perforin and/or Fas pathways (47). A classic LT-β-R-positive NK/LAK target, K562, was found to be completely resistant to the action of soluble LT-α/β₂, suggesting that neither surface LT is not involved in NK-like cytotoxicity. Genetic disruption of LT-α and the two

TNF-R does not impair CTL-mediated cell death supporting the hypothesis that TNF/LT signaling is not involved in cell–cell killing (13). Further experimental work will be required to determine whether LT signaling via direct cell–cell contact supports these conclusions.

The LT system with its heterotrimeric ligand is unusual. The cytotoxic activity resides primarily in the LT-α/β₂ form, with the LT-α/β₁ complex being much less active. The crystal structure of LT-α complexed with the TNF-α/β₂ revealed that the receptor lies in the groove between two adjacent subunits (48). The higher potency of the LT-α/β₂ form suggests that the β/β cleft, which is unique in the LT-α/β₂ heterotrimer, must interact with the LT-β-R. Biochemical analyses of this interaction have confirmed that there is a high affinity interaction of LT-β-R with LT-α/β₂ and a lower affinity interaction with LT-α/β₁ (Browning, J.L., M. Zafari, C. Benjamin, W. Meier, D. Griffiths, and K. Miatschkowski, unpublished observations). The exact nature of the signaling complex is currently unclear.

Antibodies to the TNF-R55 (49, 50), Fas receptor (21, 35, 51), CD27 (52), and CD40 (53) have been shown to have receptor-activating properties. Presumably antibodies
Table 4.  Summary of the Effects of TNF, Anti-Fas, LT-α1/β2, and Anti-LT-β-R on the Growth of Various Cells in the Presence of IFN-γ

| Name        | Type       | TNF | Anti-Fas | LT-α1/β2 | Anti-LT-β-R |
|-------------|------------|-----|----------|----------|-------------|
| Brain       |            |     |          |          |             |
| U118        | Glioblastoma | +/- | +/-      | -        | -           |
| SW1783      | Astrocytoma | -   | ++       | -        | -           |
| SW1088      | Astrocytoma | +   | +/-      | -        | -           |
| Skin        |            |     |          |          |             |
| A375        | Melanoma   | -   | -        | -        | ND          |
| SK-MEL-1    | Melanoma   | -   | -        | -        | -           |
| Colorectal  |            |     |          |          |             |
| HT29        | Adenocarcinoma | ++  | ++       | ++       | +           |
| WiDr        | Adenocarcinoma | ++  | ++       | ++       | +           |
| SK-Co-1     | Adenocarcinoma | +   | +++      | -        | -           |
| SW 403      | Adenocarcinoma | -   | +        | -        | -           |
| SW 480      | Adenocarcinoma | +   | +        | -        | -           |
| SW 620      | Adenocarcinoma | -   | -        | *‡       | -           |
| SW 837      | Adenocarcinoma | +/- | +/-      | -        | -           |
| SW 1116     | Adenocarcinoma | -   | +        | -        | ND          |
| SW 1417     | Adenocarcinoma | -   | -        | ND       | ND          |
| Colo 320DM  | Adenocarcinoma | -   | ND       | -        | ND          |
| LoVo        | Adenocarcinoma | +   | +/-      | -        | -           |
| DLD-1       | Adenocarcinoma | +   | +        | -        | +/-         |
| LS 174T     | Adenocarcinoma | ++  | ++       | -        | -           |
| LS 123      | Adenocarcinoma | +   | -        | ND       | ND          |
| T84         | Carcinoma   | +   | ++       | -        | -           |
| HCT 116     | Carcinoma   | +/- | -        | -        | ND          |
| NCI H508    | Adenocarcinoma | ++  | +        | -        | ND          |
| CACO-2      | Adenocarcinoma | -   | -        | -        | -           |
| Breast      |            |     |          |          |             |
| BT-20       | Carcinoma   | ++  | -        | -        | ND          |
| SK-BR-3     | Carcinoma   | +/- | -        | +/-      | -           |
| MCF-7       | Adenocarcinoma | ++  | +        | +/-‡     | -           |
| MDA-MB-468  | Adenocarcinoma | ++  | ND       | +/-      | +           |
| Cervix      |            |     |          |          |             |
| ME180       | Carcinoma   | +   | +        | +/-‡     | +           |
| HT-3        | Carcinoma   | ++  | -        | ++‡      | +/-         |
| MS751       | Carcinoma   | +‡  | +/-      | -        | ND          |
| Ovary       |            |     |          |          |             |
| SK-OV-3     | Adenocarcinoma | -   | -        | -        | -           |
| Pancreas    |            |     |          |          |             |
| PANC-1      | Epithelioid carcinoma | +  | +        | -        | -           |
| Capan-1     | Adenocarcinoma | -   | ++       | -        | -           |
| Capan-2     | Adenocarcinoma | -   | ++       | -        | -           |
| Lung        |            |     |          |          |             |
| A549        | Carcinoma   | +/- | ND       | -        | ND          |
| Lymphoid    |            |     |          |          |             |
| U937        | Histiocytic | ++  | +/-      | +/-      | ND          |
| K562        | Promyelocytic | -   | -        | -        | -           |

* Some growth stimulation.
‡ Altered morphology.
Heterogenous cell population, responders and nonresponders present.
Unconfirmed if LT-α component.
Figure 5. FACS analysis comparing CD40 and LT-β-R levels on three colorectal carcinoma lines, HT-29, SW620, HCT 116, and the pancreatic carcinoma CAPAN-2. Untreated cells were stained with anti-CD40 (BB20), anti-LT-β-R (BDA8), or a control mAb (MOPC 21). Control antibody staining is the curve with the lowest staining in each panel.

capable of cross-linking the receptors in the right orientation will generate receptor aggregates that can signal. Dimerization of the TNF-R55 may be sufficient to trigger TNF signaling; however, in the Fas receptor case, larger aggregates need to be formed (54). When immobilized on a plastic surface, all the anti-LT-β-R mAbs were able to induce death, including those that blocked ligand binding. Similar patterns were shown for anti-Fas receptor mAbs (51). In contrast, the anti-LT-β-R mAbs were not very effective in solution unless mAbs to two different epitopes were mixed together. In this case, it can be envisioned that aggregates larger than dimers could form, resulting in more productive signaling. When combined with the LT-α1/β2 ligand, some antibodies blocked activity, presumably by directly blocking the binding site, e.g., BDA8 and AGH1, although there was some evidence for mixed agonistic/antagonistic effects even in this case. At the other end of the spectrum, some mAbs, e.g., CDH10 and BKA11, potentiated the killing induced by ligand. Again, cross-linking of the receptor into small aggregates presumably facilitates the ability of ligand to productively cross-link and signal. Interestingly, the synergistic effect of the anti-LT-β-R mAbs with the LT-α1/β2 ligand also occurred between anti-LT-β-R mAbs and TNF. We do not have any evidence that the TNF receptors are involved in LT-β-R signaling, and therefore we interpret the effects on TNF signaling as resulting from more complex priming-like events at the intracellular level. Anti-LT-β-R mAbs can trigger NF-κB activation without inducing cell death (MacKay, F., manuscript submitted for publication), thus it is possible that both the LT-β-R and TNF-R intracellular signal transduction pathways utilize some common elements resulting in synergistic cross talk. Along with the soluble ligands and LT-β-R-Fc forms, the anti-LT-β-R mAbs are good tools for either activating or inhibiting the LT-β-R signaling pathway.

TNF can induce necrosis, apoptosis, or mixed mechanisms, depending on the cell line (55, 56), whereas Fas-triggered death is generally described as occurring by apoptosis. TNF can have either fast or slow effects on cells, probably reflecting multiple mechanisms (57). The experiments described here showed a lack of classical DNA laddering, although some DNA cleavage was detected using TUNEL techniques. The nuclei appear to condense, yet the cytoplasm balloons out in a manner characteristic of necrosis. It is possible that the ballooning occurs long after the death event and is observed in these assays, which are relatively long term compared with conventionally studied apoptotic events. Morphologically, the death induced by TNF, anti-Fas receptor, and LT-α1/β2 are similar, differing only kinetically. The signaling pathways initiated in each case may be different, as shown previously for Fas and TNF receptors (58, 59). The action of TNF is often accelerated by...
few cases in which a normal physiological signal can induce cell death as observed in vitro or whether other mechanisms are contributing. If other mechanisms are involved, a wider range of tumors may be affected in vivo than would be forecast by the in vitro analyses. Generally, more primary tumors were found to be affected by TNF in in vivo models than would be surmised from the analysis of in vitro cultured tumor lines.

An LT-β-R–based antitumor strategy may be important when considered in the context of recent advances. TNF, Fas, and LT-α/β 2 are clearly cytotoxic to tumor types with mutant p53 such as HT-29 (63), and, moreover, the TNF signaling pathway in cells of fibroblastic or epithelial origin is not sensitive to the protective effects of bcl-2 (64). Therefore, the induction of a death sequence might occur via a route that circumvents the wild-type p53 dependency of some chemotherapeutic approaches (65). A proper understanding of these signaling processes may lead to alternative strategies for controlling cancer. Clinically, isolated limb perfusion with TNF dramatically demonstrated the soundness of the approach (66), but unfortunately the systemic application of TNF was frustrated by dose-limiting toxicity resulting from activation of inflammatory cascades. Likewise, the Fas receptor is widely distributed, and anti-Fas mAbs can be very potent inducers of apoptosis; however, receptor activation also leads to rapid necrosis of normal liver cells and death in mice. This activity would certainly complicate its therapeutic application (67). The LT-β-R is present on most transformed cell types, and its activation also presents a potential anticancer therapy. Moreover, it is likely that sufficient IFN-γ exists in the tumor environment to synergize with LT-α/β 2 without exogenous administration (68). Our preliminary data indicate that LT-α/β 2 does not effectively activate primary human endothelial cells to express the VCAM or E-selectin adhesion molecules, and hamster anti-m-LT-β-R mAbs do not cause death (Hochman, P., G. Majeau, F. Mackay, and S. Browning, manuscript submitted for publication); therefore, LT-β-R signaling should lack TNF-type toxicity. If the physiological activity of LT-α/β 2 is limited to more subtle regulatory effects on lymph node function, it is conceivable that an LT-α/β 2 therapy may provide an alternative therapy for some adenocarcinomas.

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References
1. Smith, C.A., T. Farrah, and R.G. Goodwin. 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell. 76:959–962.
2. Beyaert, R., and W. Fiers. 1994. Molecular mechanisms of tumor necrosis factor-induced cytotoxicity. FEBS (Fed. Eur. Biochem. Soc.) Lett. 340:9–16.
3. Fiers, W. 1991. Tumor necrosis factor. Characterization at the molecular, cellular and in vivo level. FEBS (Fed. Eur. Biochem. Soc.) Lett. 285:199–212.
4. Tracey, K.J., and A. Cerami. 1993. Tumor necrosis factor,
other cytokines and disease. *Annu. Rev. Cell Biol.* 9:317–343.

5. Tartaglia, L.A., D.V. Goeddel, C. Reynolds, I.S. Figari, R.F. Weber, B.M. Fendly, and M.A. Palladino, Jr. 1993. Stimulation of human T-cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor. *J. Immunol.* 151: 4637–4641.

6. Pimentel-Muinos, F.X., J. Mazzana, and M. Fresno. 1995. Biphasic control of nuclear factor-kappa B activation by the T cell receptor complex: role of tumor necrosis factor alpha. *Eur. J. Immunol.* 25:179–186.

7. Paul, N.L., and N.H. Ruddle. 1988. Lymphotoxin. *Annu. Rev. Immunol.* 6:407–438.

8. Browning, J.L., A. Ngam-ek, P. Lawton, J. DeMarinis, R. Tizard, E.P. Chow, C. Hession, G.B. O’Brien, S.F. Foley, and C.F. Ware. 1993. Lymphotoxin beta, a novel member of the TNF family that forms a heteromorphic complex with lymphotoxin on the cell surface. *Cell.* 72:847–856.

9. Browning, J.L., I. Dougas, E.A. Ngam, P.R. Bourdon, B.N. Ehrenfels, K. Miatkowski, M. Zafiri, A.M. Yampaglia, P. Lawton, W. Meier, et al. 1995. Characterization of surface lymphotoxin forms. Use of specific monoclonal antibodies and soluble receptors. *J. Immunol.* 154:33–46.

10. Androlewicz, M.J., J.L. Browning, and C.F. Ware. 1992. Lymphotoxin is expressed as a heteromeric complex with a distinct 33-kDa glycoprotein on the surface of an activated human T cell hybridoma. *J. Biol. Chem.* 267:2542–2547.

11. Ware, C.F., P.D. Crowe, M.H. Grayson, M.J. Androlewicz, and J.L. Browning. 1995. Expression of surface lymphotoxin and tumor necrosis factor on activated T, B, and natural killer cells. *J Immunol.* 149:3881–3888.

12. Ware, C.F., T.L. VanAndale, P.D. Crowe, and J.L. Browning. 1995. The ligands and receptors of the lymphotoxin system. *Curr. Top. Microbiol. Immunol.* 198:175–218.

13. Abe, Y., A. Horiuchi, Y. Osuka, S. Kimura, G.A. Granger, and T. Gatanaga. 1992. Studies of membrane-associated and soluble (secreted) lymphotoxin in human lymphokine-activated T-killer cells in vitro. *Lymphokine Cytokine Res.* 11:115–121.

14. Crowe, P.D., T.L. VanAndale, B.N. Walter, C.F. Ware, C. Hession, B. Ehrenfels, J.L. Browning, W.S. Din, R.G. Goodwin, and C.A. Smith. 1994. A lymphotoxin-beta-specific receptor. *Science (Washington, DC).* 264:707–710.

15. De Togni, P., J. Goellner, N.H. Ruddle, P.R. Streeter, A. Fick, S. Mariatohan, S.C. Smith, R. Carlson, L.P. Shomick, T. Gatanaga. 1993. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science (Washington, DC).* 264:703–706.

16. Banks, T.A., B.T. Rouse, M.K. Kerley, P.J. Blair, M.L. Mucenski. 1995. Lymphotoxin-alpha-deficient mice: effects on secondary lymphoid development and humoral immune responsiveness. *J Immunol.* 155:1685–1693.

17. Miyawaki, S., N. Nakamura, H. Suzuki, M. Koba, R. Yamasu, M. Ikehara, and Y. Shibata. 1994. A new mutation, aly, that induces a generalized lack of lymph nodes accompanied by immunodeficiency in mice. *Eur. J. Immunol.* 24:429–434.

18. Pfeffer, K., T. Matsuyama, T.M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P.S. Ohashi, M. Kronke, and T.W. Mak. 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell.* 73:457–467.

19. Erickson, S.L., F.J. de Sauvage, K. Kirly, K. Carver-Moore, S. Pitts-Meek, N. Gillett, K.C.F. Sheehan, R.D. Schreiber, D.V. Goeddel, and M. Moore. 1994. Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature (London).* 372:560–563.

20. Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell.* 66:233–243.

21. Trauth, R.C., C. Kla, A.M.J. Peters, S. Matzku, P. Moeller, W. Falk, K.-M. Debain, and P.H. Krammer. 1989. Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science (Washington, DC).* 245:301–305.

22. Nagata, S., and P. Golstein. 1995. The Fas death factor. *Science (Washington, DC).* 267:1449–1458.

23. Sarin, A., M. Conen-Cibotti, and P.A. Henkart. 1995. Cytotoxic effect of TNF and lymphotoxin on T lymphoblasts. *J Immunol.* 155:3716–3718.

24. Zheng, L., G. Fisher, R.E. Miller, J. Peschon, D.H. Lynch, and M.J. Lenardo. 1995. Induction of apoptosis in mature T cells by tumour necrosis factor. *Nature (London).* 377:348–351.

25. Cleveland, J.L., and J.N. Ihle. 1995. Contenders in FasL/TNF death signaling. *Cell.* 81:479–482.

26. Funakoshi, S., D.L. Longo, M. Beckwith, D.K. Conley, G. Tsarfaty, I. Tsarfaty, R.J. Armitage, W.M. Fanslow, M.K. Spriggs, and W.J. Murphy. 1994. Inhibition of human B-cell lymphoma by CD40 stimulation. *Blood.* 83:2787–2794.

27. Smith, C.A., H.J. Gruss, T. Davis, D. Anderson, T. Farrah, E. Baker, G.R. Sutherland, C.I. Brannan, N.G. Copeland, N.A. Jenkins, et al. 1993. CD30 antigen, a marker for Hodgkin’s lymphoma, is a receptor whose ligand defines an emerging family of cytokines with homology to TNF. *Cell.* 73:1349–1360.

28. Browning, M.J., P. Krausa, A. Rowan, D.C. Bicknell, J.G. Bodmer, and W.F. Bodmer. 1993. Tissue typing the HLA-A locus from genomic DNA by sequence-specific PCR: comparison of HLA genotype and surface expression on colorectal tumor cell lines. *Proc. Natl. Acad. Sci. USA.* 90:2842–2845.

29. Miller, G.T., P.S. Hochman, W. Meier, R. Tizard, S.A. Bixler, M.D. Rosa, and B.P. Wallner. 1993. Specific interaction of lymphocyte function associated antigen-3 with CD2 can inhibit T cell responses. *J. Exp. Med.* 178:211–222.

30. Browning, J., and A. Riboloni. 1989. Studies on the differing effects of tumor necrosis factor and lymphotoxin on the growth of several human tumor lines. *J. Immunol.* 143:1859–1867.

31. Crowe, P.D., T.L. VanAndale, B.N. Walter, K.M. Dahms, and C.F. Ware. 1994. Production of lymphotoxin (LT alpha) and a soluble dimeric form of its receptor using the baculovirus expression system. *J. Immunol. Methods.* 168:79–89.

32. Johne, B., M. Gadnell, and K. Hansen. 1993. Epitope mapping and binding kinetics of monoclonal antibodies studied by real time biospecific interaction analysis using surface plasmon resonance. *J. Immunol. Methods.* 160:191–198.

33. Lee, S.H., B.B. Aggarwal, E. Rinderknecht, F. Assisi, and H. Chiu. 1984. The synergistic anti-proliferative effect of gamma interferon and tumor necrosis factor is enhanced by interferon-gamma. *Blood.* 63:175–218.

34. Deftroy, G.B., M. Naylor, W. Fiers, and F.R. Balkwill. 1987. DNA fragmentation and cytotoxicity caused by tumor necrosis factor is enhanced by interferon-gamma. *Eur. J. Immunol.* 17:689–693.

35. Yonehara, S., A. Ishii, and M. Yonehara. 1989. A cell-killing monoclonal antibody (anti-fas) to a cell surface antigen codownregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* 169:1747–1756.

36. Ashkenazi, A., S.A. Marsters, D.J. Capon, S.M. Chamow, and A. Yonehara. 1989. A cell-killing monoclonal antibody (anti-fas) to a cell surface antigen codownregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* 169:1747–1756.
49. Engelmann, H., H. Holtmann, C. Brakebusch, Y.S. Avni, I. Banner, D.W., A. D’Arcy, W. Janes, R. Gentz, H.J. Schoen-

48. Raitano, A.B., P. Scuderi, and M. Korc. 1991. Upregulation of interferon-gamma binding by tumor necrosis factor (TNF) receptor. Stoichiometry of binding to TNF alpa and TNF beta and inhibition of TNF activity. *J. Biol. Chem.* 266:18324–18329.

47. Goh, C.R., C.S. Loh, and A.G. Porter. 1991. Aspartic acid 50 and tyrosine 108 are essential for receptor binding and cytoplasmic activity of tumour necrosis factor beta (lymphotoxin). *Protein Eng.* 4:785–791.

46. Dusenbury, C.E., M.A. Davis, T.S. Lawrence, and J.W. Wilson. 1991. Induction of megabase DNA fragments by 5-fluorodeoxyuridine in human colorectal cancer tumor (HT29) cells. *Mol. Pharmacol.* 39:285–289.

45. Bortner, C.D., N.B.E. Oldenburg, and J.A. Cidlowski. 1995. The role of DNA fragmentation in apoptosis. *Trends Cell Biol.* 5:21–26.

44. Ledbetter, J., L.S. Grosmaire, D. Hollenbaugh, A. Aruffo, and S.G. Nadler. 1995. Agonistic and antagonistic properties of CD40 mAb G28-5 are dependent on binding valency. *Circ. Shock.* 44:67–72.

43. Raitano, A.B., P. Scuderi, and M. Korc. 1991. Upregulation of interferon-gamma binding by tumor necrosis factor and lymphotoxin: disparate potencies of the cytokines and modulation of their effects by phorbol ester. *J. Interferon Res.* 11:61–67.

42. Lynch, D. 1995. Biology of Fas. *Circ. Shock.* 44:63–66.

41. Crispe, I.N. 1994. Fatal interactions: Fas-induced apoptosis of mature T cells. *Immunity.* 1:347–349.

40. Hornuchi, A., Y. Abe, M. Miyake, K. Kimura, Y. Hitsumoto, N. Takeuchi, and S. Kimura. 1994. Role of membrane-associated lymphotoxin (mLT) in the killing activity of lymphotoxin-activated killer (LAK) cells towards various tumour cell lines. *Clim. Exp. Immunol.* 96:152–157.

39. Henkard, P.A. 1994. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity.* 1:343–346.

38. Banner, D.W., A. D’Arcy, W. Jans, R. Gentz, H.J. Schoenfeld, C. Broger, H. Loetscher, and W. Lesslauer. 1993. Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell.* 73:431–448.

37. Engelmann, H., H. Holtmann, C. Brakebusch, Y.S. Avni, I. Sarov, Y. Nophar, O. Hadas, O. Leitner, and D. Wallach. 1990. Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. *J. Biol. Chem.* 265:14497–14504.

36. Shalaby, M.R., A. Sundan, H. Loetscher, M. Brockhaus, W. Lesslauer, and T. Espevik. 1990. Binding and regulation of cellular functions by monoclonal antibodies against human tumor necrosis factor receptors. *J. Exp. Med.* 172:1517–1520.

35. Alderson, M.R., T.W. Toug, S. Braddy, T. Davis-Smith, E. Roux, K. Schooley, R.E. Miller, and D.H. Lynch. 1994. Regulation of apoptosis and T cell activation by Fas-specific mAb. *Int. Immunity.* 6:1799–1806.

34. van Lier, R.A.W., M.O. Pool, P. Kabel, S. Mous, F. Terpstra, M.A. De Rie, C.J. Melief, and F. Miedema. 1988. Anti-CD27 monoclonal antibodies identify two functionally distinct populations within the CD4+ T cell subset. *Eur. J. Immunol.* 18:811–816.

33. Gordon, J., M.J. Millsom, G.R. Guy, and J.A. Ledbetter. 1988. Resting lymphocytes can be triggered directly through the CDw40 (Bp50) antigen. *J. Immunol.* 140:1425–1430.

32. Dhein, J., P.T. Daniel, B.C. Trauth, A. Oehm, P. Moller, and P.H. Krummer. 1992. Induction of apoptosis by monoclonal antibody anti-APO-1 class switch variants is dependent on cross-linking of APO-1 cell surface antigens. *J. Immunol.* 149:3166–3173.

31. Laster, S.M., J.G. Wood, and L.R. Gooding. 1988. Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. *J. Immunol.* 141:2629–2634.

30. Fady, C., A. Gardner, F. Jacoby, K. Briskin, Y. Tu, I. Schmid, and A. Lichtenstein. 1995. Apoptotic cell death induced in L929 targets by exposure to tumor necrosis factor. *J. Interferon Cytokine Res.* 15:71–80.

29. Reid, T.R., F.M. Tortis, and G.M. Ringold. 1989. Evidence for two mechanisms by which tumor necrosis factor kills cells. *J. Biol. Chem.* 264:4583–4589.

28. Wong, G.H.W., and D.V. Goeddel. 1994. Fas antigen and p55 TNF receptor signal apoptosis through distinct pathways. *J. Immunol.* 152:1751–1755.

27. Grell, M., P.H. Krummer, and P. Scheurich. 1994. Segregation of APO-1/Fas antigen and tumor necrosis factor receptor mediated apoptosis. *Ear. J. Immunol.* 24:2563–2566.

26. Reid, T., P. Louie, and R.A. Heller. 1994. Mechanisms of tumor necrosis factor cytotoxicity and the cytotoxic signals transduced by the p75 tumor necrosis factor receptor. *Circ. Shock.* 44:84–90.

25. Peska, S., J.A. Langer, K.C. Zoon, and C.E. Samuel. 1987. Interferon and its actions. *Annu. Rev. Biochem.* 56:727–777.

24. Fiers, W. 1991. Tumor necrosis factor, characterization at the molecular, cellular and in vivo level. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 285:199–212.

23. Park, D.J., H. Nakamura, A.M. Chumakov, J.W. Said, C.M. Miller, D.L. Chen, and H.P. Koeffler. 1994. Transactivation and DNA binding activities of endogenous p53 in p53 mutant cell lines. *Oncogene.* 9:1899–1906.

22. Vanhaesebroeck, B., J.C. Reed, V.D. De, J. Grooten, T. Miyashita, S. Tanaka, R. Beyaert, R.F. Van, and W. Fiers. 1993. Effect of bcl-2 proto-oncogene expression on cellular sensitivity to tumor necrosis factor-mediated cytotoxicity. *Oncogene.* 8:1075–1081.

21. Lowe, S.W., H.E. Ruley, T. Jacks, and D.E. Housman. 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell.* 74:957–967.

20. Lejeune, F., D. Lienard, A. Eggermont, H. Schraffordt, B. Kroon, J. Gerain, F. Rosenkaimer, and P. Schmitz. 1994. Clinical experience with high dose tumor necrosis factor alpha in regional therapy of advanced melanoma. *Circ. Shock.* 43:191–197.

19. Ogasaawara, J., F.R. Watanabe, M. Adachi, A. Matsuzawa, T. Kasugai, Y. Kitamura, N. Itoh, T. Suda, and S. Nagata. 1993. Lethal effect of the anti-Fas antibody in mice. *Nature (Lond.).* 364:806–809.

18. Dighe, A.S., E. Richards, I.J. Old, and R.D. Schreiber. 1994. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative FNgamma receptors. *Immunity.* 1:447–456.