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Constitutive Expression of Functional 4-1BB (CD137) Ligand on Carcinoma Cells

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Members of the TNF superfamily, including Fas, Fas ligand, and CD40, have been shown to be expressed on tumor cells. In the studies described in this work, we report that another family member, the ligand for 4-1BB (CD137), is expressed on various human carcinoma cell lines, on cells of solid tumors derived from these cell lines, and cells obtained from human tumors. Expression of 4-1BB ligand (4-1BBL) mRNA was detected by both RT-PCR and Northern blot analysis, and expression of 4-1BBL protein was detected by Western blot analysis of whole cell lysates and by FACS analysis of tumor cells and cell lines. Incubation of tumor cells with a 4-1BB-Ig fusion protein led to the production of IL-8 by the cells, demonstrating that the 4-1BBL is functionally active and signals back into the tumor cells. Furthermore, 4-1BBL expressed on the carcinoma cells functioned as a costimulatory molecule for the production of cytokines (most notably IFN-γ) in cocultures of T cells and tumor cells. These findings suggest that 4-1BBL expressed on carcinoma cells may significantly influence the outcome of a T cell-tumor cell interaction. The Journal of Immunology, 2000, 165: 2903–2910.

The 4-1BB is a member of the TNF-R gene family, which includes an increasing number of proteins involved in regulation of cell proliferation, differentiation, and programmed cell death (1, 2, 3). Members include the low-affinity nerve growth factor receptor (4), TNF-R1 (5), TNF-R2 (6), CD40 (7), the Hodgkin’s Ag CD30 (8), CD27 (9), Fas/APO-1 (10), 4-1BB (11), and several others. These receptors recognize soluble or cell-bound ligands such as nerve growth factor (3), TNF (12), CD40L (13), CD27L (14), Fas ligand (FasL)3 (15), and 4-1BBL (16), which share C-terminal amino acid homology. Expression of some members of the TNF-R/ligand family on tumor cells has previously been reported (17, 18). For example, FasL on tumor cells has been implicated in the escape of tumors from immune surveillance by inducing apoptosis in tumor-infiltrating lymphocytes (19, 20).

Recently, a number of studies that used either transfected ligand (21) or a soluble form of the 4-1BB receptor to block receptor/ligand interactions (22, 23, 24) have demonstrated a role for the TNF/TNF-R family member 4-1BB/4-1BBL in T cell activation. mAbs against 4-1BB have been shown to eradicate established tumors in a mouse model (25), and to preferentially induce proliferation of CD8 T cells compared with CD4 T cells. This has led to the suggestion that 4-1BB is primarily a costimulatory molecule for CD8 T cells (26). Most recently, studies have shown that in addition to providing costimulation, 4-1BB may also promote long-term T cell survival especially of CD8 T cells (27).

It has been reported that human 4-1BBL is constitutively expressed on several types of APC, such as activated B cells, monocytes, and splenic dendritic cells, and can be induced on T lymphocytes (21, 16, 28, 29, 23). In addition, not surprisingly given their derivation, expression of 4-1BBL has also been found on tumor cells of lymphoid or myeloid origin. Studies with 4-1BBL+ APC have shown that the interaction of 4-1BB with its ligand stimulates cell proliferation and production of IL-2 and IL-4 by CD4 T cells. Moreover, it has been reported that anti-4-1BB mAb stimulate the production of IFN-γ by CD8 T cells (26). The role for 4-1BBL in the development of TH1 and TH2 cells is reported to be most apparent in the absence of a strong B7-CD28 interaction (23, 24). Likewise, Saoulli and coworkers (30) have demonstrated that isolated 4-1BBL can costimulate resting T cells via a CD28-independent pathway. Several ligands of the TNF superfamily have been shown to be able to signal in both directions, through the respective receptor and into the cell that expresses the ligand. Reverse signaling following cross-linking of 4-1BBL has been shown to inhibit proliferation, to induce apoptosis, and to up-regulate expression of Fas (CD95) on lymphocytes (31), and to stimulate macrophages to release IL-8 (32).

Although 4-1BBL is expressed on cells of hemopoietic origin, its expression on carcinoma cells has not been examined. In this study, we show that 4-1BBL is expressed to varying extents on several human carcinoma cell lines as well as on cells obtained from patient solid tumors. We demonstrate that the 4-1BBL is functional in that reverse signaling through the 4-1BBL by a 4-1BB-Ig fusion protein induces tumor cells to produce IL-8. Furthermore, we show that coculture of anti-CD3-activated T lymphocytes with tumor cells expressing 4-1BBL results in production of IFN-γ by the activated T cells.

Materials and Methods

Cell preparation and culture

Human T cells were isolated from healthy donors by standard protocols. In brief, PBMC were prepared from heparinized whole blood by Ficoll density-gradient sedimentation; T cells were then isolated by rosetting with SRBC. In some experiments, the T cells were further purified by passage through nylon wool columns or by a second round of rosetting. The human
tumor cell lines A 2780 (ovarian), Colo 205 (colon), HT 29 (colon), L 2987 (lung), LX 1 (lung), PC 3 (prostate), and HL 60 (promyelocytic leukemia) were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin. HCT 116 (colon) and SKBR 3 (breast) cell lines were grown in McCoy’s medium with the same supplements.

COS cell transfection

To obtain COS cells expressing surface 4-1BBL, the cDNA encoding for the extracellular domain of 4-1BBL (accession no. U03398) was fused to the cDNA for the intracellular and cytoplasmic domains of CD40L (accession number Z 15017) in pCDM7. COS cells were transfected by using DEAE-dextran. Briefly, cells were grown in 48-well plates, then 1 μg/ml DNA in the presence of DEAE-dextran 400 μg/ml plus Chloroquine (100 μM) in 5% NuSerum (Becton Dickinson, Bedford, MA) in DMEM (Life Technologies, Gaithersburg, MD) was added for 4 h at 37°C. The cells were then shocked with 10% DMSO in PBS. Cells were then cultured in 10% FBS/DMEM and used 48 h after transfection.

Abs, enzymes, and fusion proteins

Anti-human CD3 Ab G19.4 was obtained internally from Bristol-Myers Squibb (New York, NY). Goat anti-rabbit FITC conjugate was from BioSource International (Camarillo, CA). Goat anti-human PE conjugate was from Jackson ImmunoResearch (West Grove, PA). Mouse anti-human HLA class I PE, rat anti-mouse CD45 PE conjugate, and the isotype controls rat IgG2b PE conjugate and mouse IgG1 PE were from PharMingen (San Diego, CA). The nonstimulatory anti-CD137 Ab clone BBK-2 was from NeoMarkers (Francisco, CA); the clone 4B4-I-1 was from Ancell (Bayport, MN). All other reagents were obtained from Sigma (St. Louis, MO).

Rabbit polyclonal antisera to 4-1BBL were prepared by immunizing New Zealand white rabbits with the peptide CHTEQARAHWQLTQ representing aa 217–230 of the 4-1BBL polypeptide sequence, conjugated to keyhole limpet hemocyanin. This sequence of the peptide is predicted to form part of the extracellular domain of 4-1BBL. Polyclonal antisera were purified by affinity chromatography on protein A-Sepharose. After elution, the antisera were dialyzed into PBS, filter sterilized (0.22 μm), and stored at 4°C. The soluble 4-1BB fusion protein (human 4-1BB with a human IgG1 tail) (33) and soluble B7-2 fusion protein (human B7-2 with a human IgG1 tail) (34) were constructed and prepared as previously described.

Disaggregation of solid tumor tissue

Tumors were excised and pooled, and 2–3 g of tumor tissue was minced in 7 ml HBSS. A tumor cell suspension was obtained by adding enzymes to a final concentration of 0.02% DNase, 0.3% collagenase, and 0.4% hyaluronidase, and incubating for 2 h at 37°C. Cells were washed three times in HBSS and passed three times through a 25-gauge needle. After centrifugation, cells were resuspended in RPMI 1640 containing 10% FCS.

Flow cytometry

For the FACS analysis of 4-1BBL expression on carcinoma cell lines, cells were washed once in PBS and 105 cells were incubated at 4°C in 100 μl staining buffer (RPMI 1640 medium, 2% FCS, 2.5 mM EDTA, mouse IgG at 250 μg/ml) containing 10 μg/ml of 4-1BBL-Ab or 10 μg/ml B7-2-Ig fusion proteins. After incubation, cells were washed twice with PBS and incubated at 4°C for 30 min in 100 μl of a 10 μg/ml solution of PE-conjugated goat anti-human IgG Ab in staining buffer and then washed further. Alternatively, 10 μg/ml rabbit anti-4-1BBL polyclonal antisera or 10 μg/ml rabbit IgG followed by 100 μl of 20 μg/ml goat anti-rabbit FITC-conjugated Abs were used under the same conditions. When the rabbit antisera was used, cells were counterstained with propidium iodide, and positive cells were gated out to ensure only live cells were evaluated. For analysis of cells derived from solid tumors growing in athymic mice, 106 cells were stained with anti-4-1BBL rabbit polyclonal antisera or control rabbit IgG, as described above. After washing twice in PBS, a second staining step was incorporated using 10 μg/ml mouse anti-human HLA class I PE conjugate, or 10 μg/ml mouse IgG1 PE as the isotype control, in 100 μl of cell suspension. With this procedure, by gating on the HLA-positive cells, only expression were seen on the HCT 116 and Colo 205 cell lines (Fig. 1A, panels 2 and 3), with lower levels in HT 29 cells (Fig. 1A, panel 4). Very similar results were found when the cells were analyzed with a polyclonal rabbit anti-4-1BBL antisera compared with normal rabbit IgG (Fig. 1B). A previous report indicated that 4-1BBL was expressed on T lymphocytes only following stimulation (16). In concurrence, no expression could be detected on extraction of mRNA, the Fast Track 2.0 kit from Invitrogen (Carlsbad, CA) was used as instructed by the manufacturer. The cDNA library for human small intestine was internally obtained from Bristol-Myers Squibb.

RT-PCR

cDNA was prepared using the Superscript Preamplification System for First Strand cDNA Synthesis (Life Technologies, Rockville, MD) using 3 μg total RNA per sample. Aliquots of 5 μl of the cDNA were then amplified with 44 μl of PCR supermix high fidelity (Life Technologies) and 0.5 μl of each primer (final concentration, 0.5 μM). For detection of human 4-1BBL cDNA, the primers used for amplification were human 4-1BBL sense primer corresponding to nucleotides 484–513 (5'-GCT TCA TTT GCG CGC TTC CCA CTA CTA GTC-3') and antisense primer complementary to nucleotides 926–949 (5'-GGC TCT AGA TAT CAA GGT CCA ACT TGG GGA AGG-3'). Primers for human β-actin (5'-GTG GGG CGC CCC AGG CCC AC-3', nucleotides 144–163 and 5'-CTC ATT GTC AGC CAG CAT GC-3', nucleotides 660–683) served as control. After denaturation at 94°C for 5 min, 40 PCR cycles were performed, each consisting of a denaturation step (94°C, 1 min), an annealing step (52°C, 2 min), and an elongation step (72°C, 2 min). The PCR products (expected size 465 bp for human 4-1BBL, 539 bp for human actin) were separated by electrophoresis on 1% agarose gel and visualized by staining with ethidium bromide.

Northern blot hybridization

Samples of mRNA (2 μg) were fractionated according to size in 3% formamide/1.2% agarose gels and then transferred to nylon membranes. The membranes were prehybridized for 1 h in hybridization solution (Express hyb; Clontech, Palo Alto, CA) at 65°C, then hybridized for 2 h with a random primed [32P]dCTP-labeled (Random Primed DNA Labeling Kit; Boehringer Mannheim, Indianapolis, IN) human 4-1BBL cDNA fragment or a GAPDH cDNA fragment as a control (2 × 106 cpm/ml). Membranes were washed twice in 2 × SSC with 0.05% SDS at room temperature and twice in 0.1 × SSC with 0.1% SDS at 65°C and exposed to x-ray film with intensifying screens at ~70°C.

Western blot analysis

Tumor cells were isolated by dissociation with RPMI 1640 containing 5 mM EDTA and washed twice with PBS, and the cell pellets were then frozen at ~70°C. Cell pellets (3 × 1010 total cells) were lysed at 1 × 1010 cells/ml in cell lysis buffer (33) containing 1 Triton X-100 and protease inhibitors. Cell lysates were clarified by centrifugation at 16,000 g for 20 min, and an equal volume of 2 × SDS Laemmli sample buffer containing 2-ME. Samples were heated at 95°C for 5 min before separation by SDS-PAGE on 8–16% acrylamide gels. Gels were then transferred to polyvinylidene difluoride membranes by Western blotting; the membranes were blocked with buffer containing 5% BSA and 1% nonfat dried milk, probed with anti-4-1BBL polyclonal antisemum (1 μg/ml), and visualized by probing with HRP goat anti-rabbit IgG and Western blot chemiluminescence reagent (NEN Life Science Products, Boston, MA).

ELISA

Cytokine assays were performed by ELISA according to the manufacturer’s instructions using OptEIA sets from PharMingen. Cytokine concentrations in supernatants are expressed as mean ± SEM of quadruplicates. Where indicated, results were compared using the Student t test.

Results

Expression of 4-1BBL on carcinoma cells

The expression of 4-1BBL on the surface of the cell lines A 2780, Colo 205, HCT 116, HT 29, LX 1, PC 3, and SKBR 3 was investigated by flow cytometry. Analysis of the staining of cells with a human 4-1BB-human IgG1 Fc fusion protein (4-1BBig) compared with a B7-2-Ig fusion protein (B7-2ig) revealed that the cells expressed 4-1BBL at various densities. The highest levels of expression were seen on the HCT 116 and Colo 205 cell lines (Fig. 1A, panels 2 and 3), with lower levels in HT 29 cells (Fig. 1A, panel 4). Very similar results were found when the cells were analyzed with a polyclonal rabbit anti-4-1BBL antisera compared with normal rabbit IgG (Fig. 1B). A previous report indicated that 4-1BBL was expressed on T lymphocytes only following stimulation (16).
FITC-conjugated goat anti-rabbit polyclonal Ab. Cells were counterstained with propidium iodide to exclude dead cells. The cell lines were as given in A lines were as follows: panel 1 Ig (open histograms), followed by staining with a secondary PE-conjugated goat anti-human Ig polyclonal Ab. Cell panels 1, Resting peripheral T cells from two different donors (open histograms) or purified rabbit IgG (rabbit anti-human 4-1BBL polyclonal antiserum, as described in B. D, Solid tumors from athymic mice were disaggregated and then stained with either anti-4-1BBL polyclonal antiserum or rabbit IgG together with an anti-human HLA class I PE conjugate for the selection of human cells. Gates were set to include only the HLA-positive cells in the analysis. Tumor cells were as follows: panel 1, HCT 116; 2, L 2987; 3, LX 1, 4, taxol-resistant human ovarian tumor; 5, human pancreatic tumor.

To confirm the expression of 4-1BBL on tumor cell lines, total RNA was extracted from the cells and 4-1BBL expression was evaluated using RT-PCR. Each of the tumor cell lines expressed mRNA for 4-1BBL, as revealed by the expected products of 465 bp (Fig. 2A). The level of expression of 4-1BBL differed on the various cell lines. The RT-PCR results were confirmed by Northern blot analysis using the cDNA encoding for the extracellular domain of 4-1BBL as a hybridization probe. One larger and two smaller mRNA transcripts of sizes 4.4, 3, and 1.6 kb were found, similar to that previously described (16). The level of mRNA expression was highest in HCT 116 and Colo 205 cell lines, in accordance with the results obtained in the FACS analysis (Fig. 2B).

The expression of 4-1BBL protein was determined by Western blot analysis of Triton X-100 detergent extracts of the tumor cells (1 × 10⁶/ml). A major protein band of about 60 kDa was detected using the IgG fraction of the anti-4-1BBL antiserum. The 4-1BBL was detected in all of the cell lines, with the highest expression in the extract from HCT 116 cells (Fig. 2C, lane 3). No band was detected in extracts from unactivated peripheral T cells. The antiserum also recognized soluble 4-1BBL expressed as a fusion protein with a mouse CD8 tag (data not shown).

**Cytokine release of carcinoma cells induced by reverse signaling through 4-1BBL**

It has been reported that ligation of 4-1BBL can activate macrophages and induce cytokine production (32). To determine whether 4-1BBL expressed on the tumor cell lines was functionally active, the cells were incubated for 24 h in the presence of 4-1BBIg, B7-2Ig, or in medium alone. B7-2Ig did not enhance IL-8 secretion

**FIGURE 1.** FACS analysis of the expression of 4-1BBL. A, A total of 1 × 10⁶ cells of the different carcinoma cell lines was stained with human 4-1BB Ig (shaded histograms) or human B7-2Ig (open histograms), followed by staining with a secondary PE-conjugated goat anti-human Ig polyclonal Ab. Cell lines were as follows: panel 1, A 2780; 2, Colo 205; 3, HCT 116; 4, HT 29; 5, LX 1; 6, PC 3; 7, SKBR 3. B, A total of 1 × 10⁶ cells was stained with rabbit anti-human 4-1BB Ig polyclonal antiserum (shaded histograms) or purified rabbit IgG (open histograms), followed by staining with a secondary FITC-conjugated goat anti-rabbit polyclonal Ab. Cells were counterstained with propidium iodide to exclude dead cells. The cell lines were as given in A. C, Resting peripheral T cells from two different donors (panels 1 and 2) were stained as described in B; Colo 205 (panel 3) and HCT 116 (panel 4) cells were stained with rabbit anti-4-1BB Ig after prior addition of 4-1BBIg (dotted line) or B7-2Ig (shaded histograms), as described in B. D, Solid tumors from athymic mice and the resulting xenografts were excised, the cells were cultured in athymic mice with the anti-4-1BB Ig antiserum revealed expression of 4-1BBL on tumor cells derived from patient tumors and cultured in athymic mice with the anti-4-1BB Ig antiserum. One larger and two smaller mRNA transcripts of sizes 4.4, 3, and 1.6 kb were found, similar to that previously described (16). The level of mRNA expression was highest in HCT 116 and Colo 205 cell lines, in accordance with the results obtained in the FACS analysis (Fig. 2B).
over that found in untreated cells. Treatment of the cells with 4-1BBIg induced a marked increase in the levels of IL-8 in the cell supernatants from Colo 205 (4.2-fold), HCT 116 (2.2-fold), and LX 1 (2.4-fold); LX 1 cells were of note because they produced high spontaneous levels of the cytokine, and this was further stimulated. However, the largest increase induced by 4-1BBIg was seen with Colo 205 cells (Fig. 3A). Very little effect of 4-1BBIg on IL-8 secretion was observed in A 2780 and SKBR 3 cell lines. PC 3 cells exhibited very high levels of constitutive production of IL-8 that was not increased further by 4-1BBIg (data not shown). In contrast to the effect on IL-8, treatment of HCT 116, LX 1, or Colo 205 cells with 4-1BBIg did not significantly stimulate the release of IL-6, IL-10, IL-12, TNF-α, or TGF-β into the cell supernatants (data not shown). Stimulation of HCT 116 and LX 1 cells for 24 h with increasing concentrations of 4-1BBIg showed that IL-8 production was dose dependent (Fig. 3B). Addition of the anti-4-1BB mAb BBK-2 or 4B4-1 (20 μg/ml) to the cultures before stimulation of the cells with 4-1BBIg significantly (all p < 0.03) reduced the stimulation of IL-8 release (Fig. 3C), whereas control mouse IgG1 was without effect. This confirms that the 4-1BBIg-induced IL-8 production from the carcinoma cells is mediated through the 4-1BBL/4-1BB receptor interaction.

**FIGURE 2.** Expression of 4-1BBL mRNA and protein. A, RT-PCR analysis of 4-1BBL mRNA expression in tumor cell lines. Expression was determined by analysis of an equal level of mRNA isolate from each cell line. HL 60 cell cDNA was used as positive and human small intestine cDNA as negative control. The mRNA-specific amplification products for 4-1BBL and actin were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. The figure shown is a reverse image. Lanes 1–7, correspond to the following tumor cells: 1, A 2780; 2, Colo 205; 3, HCT 116; 4, HT 29; 5, LX 1; 6, PC 3; 7, SKBR 3.

**B**

GAPDH

4-1BBL

A 2780, Colo 205, HCT 116, HT 29, LX 1, PC 3, 7, SKBR 3.

**C**

Western blot analysis of 4-1BBL expression in tumor cell lines. Cells were lysed in Triton X-100 buffer at 10 mg/ml, separated by SDS-PAGE on 8–16% polyacrylamide gels, and transferred to polyvinylidene difluoride membranes; the membranes were blocked and then probed with the monoclonal anti-4-1BB Ab, followed by goat anti-rabbit IgG-HRP conjugate. Lanes: 1, A 2780; 2, Colo 205; 3, HCT 116; 4, HT 29; 5, LX 1; 6, PC 3; 7, SKBR 3.

**4-1BBL-expressing carcinoma cells induce T cells to produce IFN-γ**

In addition to showing that the tumor cells expressed 4-1BBL that was capable of signaling back into the tumor cells, it was important to determine whether 4-1BBL expressed on the tumor cells was able to activate the costimulatory 4-1BB pathway of human T cells. One response of T cells to ligation of 4-1BB, in conjunction with signals through the TCR, is the production of IFN-γ (26). Thus, the effect of carcinoma cells on T cell production of IFN-γ was determined. HCT 116 cells were cultured in 96-well plates, and human T cells were added to some of the cultures, with or without addition of soluble anti-CD3. After 48 h, the culture supernatants were harvested and analyzed for production of IFN-γ by ELISA. In the presence of soluble anti-CD3, T cells alone or T cells cocultured with HCT 116 cells produced very little IFN-γ (2.1 pg/ml ± 0.8 and 13.5 pg/ml ± 3.9, respectively). In the presence of soluble anti-CD3, the HCT 116 cells alone only produced very low levels of the cytokine (1.9 pg/ml ± 0.4). Similarly, low levels of IFN-γ (5.9 pg/ml ± 1.7) were produced when T cells alone were cultured with soluble anti-CD3. In contrast, cocultures of tumor cells with T cells in the presence of soluble anti-CD3 induced marked increases in the levels of IFN-γ (94.6 pg/ml ± 27.5; Fig. 4A).

FACS analysis had revealed that the carcinoma cell lines HCT 116 and HT 29 express 4-1BBL at different densities, with HCT 116 expressing higher levels than HT 29 cells. Thus, equivalent numbers of HCT 116 and HT 29 were cultured in 96-well plates and human T cells were added together with soluble anti-CD3. The cultures were incubated for 48 h, and supernatants were harvested and analyzed for production of IFN-γ by ELISA. The level of IFN-γ found in the coculture supernatants of both HCT 116 and HT 29 cells was dependent on the number of tumor cells present in the culture. By increasing the number of HCT 116 cells from 6.25 × 10⁵ to 2 × 10⁶ cells in the coculture experiments, the level of IFN-γ (over that in T cells alone) was raised from 2- to about 20-fold, respectively. The same experiment with HT 29 cells resulted in a 1- to 5-fold increase, respectively (Fig. 4B). In wells with 2 × 10⁵ tumor cells, the levels of the cytokine were significantly (p < 0.01) lower with the HT 29 cells (21.4 pg/ml ± 16.1) than with the HCT 116 cells (100.7 pg/ml ± 29.2, respectively). Stimulation of T cells with 2 × 10⁵ tumor cells gave a 17-fold increase of IFN-γ levels using HCT 116 cells, and a 5-fold increase using HT 29 cells, compared with that from T cells in the absence of tumor cells. LX 1, which we showed to have intermediate levels of 4-1BBL surface expression, stimulated a 7-fold increase in the levels of IFN-γ (data not shown). Together, these results indicate that under the conditions used, there appears to be a dose-dependent relationship among the density of 4-1BBL expressed on tumor cells, the number of cells, and the levels of IFN-γ production.
The requirement for 4-1BBL in these cultures was verified by culturing HCT 116 cells with T cells and anti-CD3 in the absence or presence of two different nonstimulatory anti-4-1BB mAb, BBK-2, and 4B4-1. In the absence of tumor cells, T cells cultured with anti-CD3 together with either of these two anti-4-1BB mAb did not produce IFN-γ, whereas IFN-γ production was stimulated with several other anti-4-1BB mAb (data not shown). Cocultures were set up as described, but in the presence of 10 μg/ml of the BBK-2 or 4B4-1 anti-4-1BB mAb or purified mouse IgG. After 48 h, supernatants were harvested and assayed by ELISA. Production of IFN-γ was significantly inhibited by both BBK-2 (p < 0.01) and 4B4-1 (p < 0.05) mAb, whereas addition of mouse IgG was without effect (Fig. 4C).

To additionally verify that the induction of IFN-γ production was due to expression of 4-1BBL on the tumor cells, COS cells growing in 48-well plates were transfected with CDNA to obtain 4-1BBL-expressing cells. After 48 h, the plates were washed and medium alone or medium containing 2 × 10^5 T cells was added to transfected or mock-transfected cells; additionally, anti-CD3 Ab (2 μg/ml) was added to each well. After an additional 48 h, supernatants were harvested and analyzed by ELISA. Compared with mock-transfected cells, COS cells transfected with 4-1BBL showed significantly (p < 0.01) enhanced IFN-γ production (11.3 pg/ml + 10.3 vs 164.3 pg/ml + 47.3, respectively). This increase was markedly inhibited (p < 0.02) by addition of the nonstimulatory anti-4-1BB mAb BBK-2 (44.1 pg/ml + 18.4) to the cultures (Fig. 4D).

**Discussion**

Several reports have suggested an important role for 4-1BB in the stimulation or costimulation of T cells (22–30, 36). These results indicate that 4-1BB is primarily a costimulatory molecule for CD8 T cells (26) that functions independently of CD28. In addition, 4-1BB may also promote long-term T cell survival (27). Expression of 4-1BBL has been reported on lymphocytes, macrophages, and cell lines of lymphoid and myeloid origin (22–30, 32).

In the study reported in this work, we show that the 4-1BBL is expressed and functional on carcinoma cells. Analysis by FACS revealed expression of 4-1BBL at different densities on several different carcinoma cell lines, on cells of human tumor cell lines growing as solid tumors in athymic mice, and on human tumor cells derived from patient samples after biopsy and propagation in athymic mice. In human tumors, infiltrating APC or lymphocytes might be expected to express 4-1BBL, which would complicate detection of 4-1BBL expression on the carcinoma cells. Thus, the human tumors were propagated in athymic mice to remove human
APC. To ensure that only human tumor cells were investigated for expression of 4-1BBL and not, for example, tumor-infiltrating mouse lymphocytes, cells were also stained with mAb for human HLA class I. Only HLA class I-positive cells were evaluated. Similar results were obtained if the cells were stained with anti-4-1BBL and anti-mouse CD45, and the population expressing CD45 was gated out. Expression of 4-1BBL in tumor cells was confirmed by RT-PCR analysis of total RNA and Northern blot analysis of mRNA extracted from tumor cells, and additionally by Western blot analysis of tumor cell lysates.

There have been reports of bidirectional signaling for several ligands of this family (37, 38), and recent studies have addressed the effects of reverse signaling through 4-1BBL. Cross-linking of 4-1BBL expressed on T lymphocytes inhibited the proliferation of the cells and induced apoptosis in activated T cells in a Fas-independent manner (31). Reverse signaling through 4-1BBL has also been observed in monocytes, and this has been shown to deliver a potent survival signal and to induce activation of the cells to release IL-8 (32).

There are several reports indicating that both colonic epithelial cells and other epithelial cells such as bronchial epithelia and gastric carcinoma cells can produce IL-8 (40). In our experiments, activation of the tumor cells with 4-1BBIg enhanced IL-8 production from the tumor cells. This cytokine is a potent neutrophil, T cell, and basophil chemoattractant, and release of IL-8 is thought to amplify an ongoing acute immune response (39). Thus, it is likely that reverse signaling through 4-1BBL on the carcinoma cells may influence the immune response at the site of the tumor. There did not appear to be a direct correlation between the levels of 4-1BBL expression on tumor cells and the IL-8 production induced by 4-1BBIg. This might be due to a differing ability of the 4-1BBIg on the tumor cells to transduce the appropriate signal into the cell after engagement with 4-1BBL. At present, the mechanism for 4-1BBL signaling back into cells is unknown.

FIGURE 4. 4-1BBL expression on carcinoma cells induces IFN-γ production from human T cells in coculture. A, HCT 116 cells were grown for 48 h in 96-well plates. After the exchange of media, 1 x 10^5 human T cells were added together with 2 μg/ml anti-CD3 mAb G 19.4, where indicated. Plates were incubated for 48 h, and the supernatants were then harvested and assayed by ELISA. Additionally, HCT 116 cells and T cells alone or together ± anti-CD3 mAb were cultured as indicated. Samples were assayed in quadruplicate. The data shown, mean with SD, are from one experiment. Similar results were obtained with T cells from eight different donors each in two independent experiments. B, HCT 116 or HT 29 cells were plated at the indicated density and grown for 48 h in 96-well plates. After exchange of medium, primary human T cells (1 x 10^6/well) and 2 μg/ml CD3 Ab were then added. Plates were incubated for 48 h, then supernatants were harvested and quantitated by ELISA. Samples were assayed in quadruplicate. Similar results were obtained with T cells from six different donors in two independent experiments each. C, Equal numbers of HCT 116 cells were grown on 96-well plates, and the culture medium was then discarded and 1 x 10^5 T cells/well in fresh medium alone or medium containing anti-4-1BB mAb BBK-2, 4B4-1, or purified mouse IgG, at a final concentration of 10 μg/ml together with anti-CD3 Ab to a final concentration of 2 μg/ml were added. Supernatants were harvested after 48 h and quantitated in quadruplicate by ELISA. D, COS cells growing in 48-well plates were transfected with cDNA encoding for the extracellular domain of 4-1BBL fused to the cDNA for the intracellular and cytoplasmic domains of CD40L (COS*). Control cells were treated identically, except that no cDNA was added (COS). After 48 h, the plates were washed twice with PBS and medium alone vs medium containing 2 x 10^6 T cells was added to transfected and untransfected cells. Anti-CD3 mAb to a final concentration of 2 μg/ml was added to each well. After 48 h, supernatants were harvested and assayed in quadruplicate by ELISA after 48 h.
The ability of 4-1BBL expressed on the carcinoma cells to deliver a signal to T cells through 4-1BB was also evaluated. Because it has been reported that T cells treated with anti-CD3 and anti-4-1BB mAb produce IFN-γ (26), the ability of the tumor cells to stimulate T cells was assessed. Purified peripheral T cells were cocultured with HCT 116 cells, which showed high expression of 4-1BBL, and this resulted in the release of IFN-γ into the culture medium. Similar but more variable results were obtained for the induction of IL-2 (data not shown). Monocultures of T cells or tumor cells, with or without anti-CD3, produced only low levels of IFN-γ. Similarly, only low levels of IFN-γ were found in supernatants of cocultures of T cells and tumor cells in the absence of anti-CD3. This suggests that 4-1BBL on the carcinoma cells was able to deliver a costimulatory signal, which also required activation of the TCR. A comparison between IFN-γ induction in the coculture with the HT 29 tumor cell line (low expression of 4-1BBL), LX 1 cells (intermediate expression), and HCT 116 cells (high expression) showed that the HCT 116 cells induced the most of cytokine, followed by LX 1 and HT 29, indicating that IFN-γ production was related to the level of 4-1BBL expressed on tumor cells. Furthermore, IFN-γ induction was dose dependent on the number of the tumor cells in the cocultures.

The level of IFN-γ produced by the T cells was markedly reduced by the addition of neutralizing anti-4-1BB mAb to the cultures, which suggests that binding of 4-1BBL was indeed responsible for the induction of the cytokine in the cultures. Other 4-1BB mAb enhanced rather than inhibited IFN-γ production from isolated T cells (data not shown); most likely, these mAb are able, even when soluble, to activate directly the 4-1BB receptor on T cells. No expression of the costimulatory molecules B7-1 and B7-2 could be detected on the HCT 116 cells by FACS analysis. In addition, incorporation of B7-Ig into the cultures had no effect on IFN-γ release (data not shown). These results indicate that the observed IFN-γ release of T cells most likely was not due to a pathway involving CD28/B7.

The ability of 4-1BBL on carcinoma cells to stimulate the T cells was confirmed by the transfection of COS cells with a construct containing the extracellular domain of 4-1BBL, with the membrane spanning and cytoplasmic domains of CD40L. In the presence of anti-CD3, these transfectants, unlike the mock transfectants, were able to stimulate production of IFN-γ in a T cell coculture assay. This induction of IFN-γ by the 4-1BBL-transfected COS cells was greatly reduced by addition of neutralizing anti-4-1BB mAb, confirming that 4-1BBL was responsible for the activation of the T cells.

The delivery of a costimulatory signal has been shown to be critical for the activation of T cells and for the prevention of induction of either cell death or an anergic state. Previous studies have shown that, while unprimed T cells require 4–5 days to respond to anti-4-1BB Abs, only 24 h are required for T cells to produce IL-2 in response to 4-1BBL in the absence of CD28 costimulation (30). This suggests that the natural ligand for 4-1BB provides a more potent signal than that delivered by Ab ligation. In our experiments, we were able to detect elevated levels of IFN-γ in supernatants of resting T cells cocultured with the 4-1BBL-expressing carcinoma cells in the presence of anti-CD3 as early as after 16 h, while strongest stimulation was seen after 48 h (data not shown). A relatively high concentration of anti-CD3 (2 μg/ml) was needed in these experiments to provide optimal stimulation. Saoulli and coworkers (30) have shown that with optimal signaling through the TCR, 4-1BBL was as effective as anti-CD28 Ab, while at low doses of anti-CD3, anti-CD28 was more effective.

The expression and role of other members of the TNF family in tumor cell function have been intensively discussed in the recent years (e.g., Refs. 17 and 18). Many reports have addressed the role of FasL on tumor cells in host-tumor interaction (e.g., Refs. 19 and 20), and these reports indicate that in some situations, FasL expressed on tumors may be involved in the escape of tumors from immune surveillance. The finding that carcinoma cells can express 4-1BBL indicates that tumor cell/T cell interactions are indeed very complex. The role of 4-1BBL on carcinoma cells remains to be fully elucidated. While treatment of tumor cells with 4-1BBLg increased IL-8 production, preliminary results showed that this treatment had no major effect on the expression of 4-1BBL on the cells. The effect of ligation of 4-1BBL on the tumor cells on subsequent MHC and costimulatory molecule expression and Ag presentation to T cells is currently under study. Among the various carcinoma cells, we have observed significant differences in the levels of MHC class I, CD80, and CD86. Preliminary analysis has revealed no major changes in the levels of these surface molecules following stimulation of the cells with 4-1BBLg; however, this is the subject of further studies.

Stimulation of IFN-γ production in T cells by the tumor cells through 4-1BB might be expected to both increase MHC expression and to enhance T cell cytolytic activity, thus stimulating an immune response. Indeed, Melero and coworkers (41) and Guinn and coworkers (42) have shown that transfection of 4-1BBL into mastocytoma or lymphoma cells resulted in protective immunity against these tumors. Thus, it is unclear why carcinoma cells would express 4-1BBL. It is possible that down-regulation of the expression of the 4-1BBL occurs within the tumor; however, this seems unlikely because surface expression was detected on cells from human tumors. Possibly, 4-1BBL signaling back into the tumor cell may stimulate an as yet unrecognized pathway, which is advantageous for the growth or survival of the carcinoma cell. Although reverse signaling through 4-1BBL induces apoptosis in T lymphocytes in a Fas-independent manner (31), we did not see any induction of cell death in the cell lines mediated by 4-1BBLg under the specified culture conditions (data not shown).

The overall outcome of the interaction between T cells and solid tumors is dependent on the interplay between cell surface receptors and their ligands on the tumor cells. Our results indicate that the 4-1BB receptor/ligand system is likely to play a significant role in this host-tumor interaction.

References


