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_J Immunol_ 2002; 168:4897-4906; doi: 10.4049/jimmunol.168.10.4897
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4-1BB Ligand-Mediated Costimulation of Human T Cells Induces CD4 and CD8 T Cell Expansion, Cytokine Production, and the Development of Cytolytic Effector Function

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4-1BB (CD137) is a costimulatory member of the TNFR family expressed on activated T cells. Its ligand, 4-1BBL, is expressed on activated APC. In the mouse, CD8 T cells are preferentially activated by agonistic anti-murine 4-1BB Abs. However, murine 4-1BBL can stimulate both CD4 and CD8 T cells. To date, there are only limited data on the effects of 4-1BBL on human T cell responses. To further understand the role of 4-1BBL in human T cell responses, we compared human CD4 and CD8 T cell responses to transduced human 4-1BBL plus TCR-mediated stimulation. Both human CD4 and CD8 T cells responded to 4-1BBL. The presence of 4-1BBL on the APC led to increased expansion, cytokine production, and the development of cytolytic effector function by human T cells. In unfractinated T cell cultures, CD4 and CD8 T cells could expand to a similar extent in response to signals through the TCR and 4-1BB, as assessed by CFSE labeling and by quantitating T cell numbers in the cultures. In contrast to the results with total T cells, isolated CD8 T cells produced less IL-2 and expanded to a lesser extent than isolated CD4 T cells responding to 4-1BBL. Thus, 4-1BBL is most effective when both CD4 and CD8 T cells are included in the cultures. CD28 and 4-1BB were found to synergize in the induction of IL-2 by human T cells, and CTLA-Ig partially blocked 4-1BBL-dependent IL-2 production. However, a portion of the 4-1BBL-mediated effects were independent of CD28-B7 interaction. The Journal of Immunology, 2002, 168: 4897-4906.

4-1BB is a costimulatory member of the TNFR family, expressed on activated CD4 and CD8 T cells (for review, see Refs. 1 and 2). Its ligand, 4-1BBL, is expressed on activated APC, including IFN-γ-activated macrophages, Ig or CD40 ligand-activated B cells, or dendritic cells (3-5). There is an accumulating body of literature indicating that murine 4-1BB can augment T cell proliferation, cytokine production, and cytolytic effector function and prevent activation-induced cell death (1, 2). When given in conjunction with a strong signal through the TCR, 4-1BBL can induce IL-2 production by resting CD4 T cells independently of the CD28 costimulatory pathway (5, 6). However, when signals through the TCR are limiting, CD28 is much more effective in costimulation of IL-2 production by resting T cells than 4-1BB, probably due to the requirement for 4-1BB to be induced on the T cell by the TCR signal (6). Although some studies have suggested a preferential role for 4-1BB in CD8 T cell activation (7, 8), a number of studies have shown that CD4 T cells also respond to 4-1BBL (5, 9-12). Recent experiments have shown that murine CD4 and CD8 T cells respond to 4-1BBL-mediated costimulation to a similar extent in terms of T cell expansion (13). In contrast, agonistic anti-4-1BB Abs differentially stimulate the two subsets both in vitro and in vivo (7, 8). In vivo, the 4-1BB/4-1BBL costimulatory pathway has been shown to augment secondary CTL responses to influenza virus and lymphocytic choriomeningitis virus (14-16) as well as augment anti-tumor immunity (17-21). Doubly deficient, CD28−/−/4-1BB−/− mice show a delay in skin allograft rejection compared with mice lacking either one of these costimulatory molecules (14). In mouse models of graft-vs-host disease, 4-1BB and 4-1BBL have been shown to play a role in both the MHC class II- and MHC class I-restricted components of the response (12).

Human 4-1BB (CD137) was cloned by three different groups (22-24) and has also been referred to as ILA (24). 4-1BB/ILA is 60% identical with murine 4-1BB. It contains notable differences in its cytoplasmic tail from murine 4-1BB. In particular, the single tyrosine residue in the cytoplasmic domain of 4-1BB is found at position 220 of human 4-1BB and at position 254 of murine 4-1BB. Human 4-1BB also diverges from murine 4-1BB at the putative Lck binding site (25), with 4-1BB expressing the CXCP Lck binding motif, whereas in human 4-1BB this sequence is altered to CXFP. Both human and murine 4-1BB have in common two sites for binding TNFR-associated factor 2 (26-28), an adapter protein that is essential for mediating downstream signaling events leading to IL-2 production in response to 4-1BB signaling (6, 29, 30). Human 4-1BB is expressed on activated CD4 and CD8 T cells and is expressed at higher levels on activated CD8 T cells from HIV+ individuals than on CD8 T cells from healthy donors (17). In addition to its expression on T cells, human 4-1BB expression has been reported on epithelial and hepatoma cells (24) as well as on blood vessels from individuals with malignant tumors (31). Interestingly, the 4-1BB gene maps to human chromosome 1p36, a region previously associated with several malignancies (32). A soluble form of 4-1BB has also been reported in the serum of patients with rheumatoid arthritis (33). Human monocytes express 4-1BB, and anti-4-1BB has been shown to augment TNF-α and IL-8 production by monocytes (34). 4-1BB is also expressed on neutrophils, and anti-4-1BB can ameliorate activation-induced cell death of neutrophils (35).
4-1BB-transfected CV1 cells or anti-4-1BB Abs can augment PHA-stimulated or CD3-stimulated T cell proliferation, respectively (22, 36). For a human Th1 clone, the effects of anti-4-1BB were only observed in conjunction with CD28 signaling, and 4-1BB was found to enhance proliferation and cytokine production by the anti-CD3- and anti-CD28-stimulated cells (37). In contrast, murine 4-1BB-mediated costimulation is CD28 independent (5).

Human 4-1BBL is found on EBV-transformed human B cell lines as well as on the monocyte cell line THP-1. It shares 36% identity with murine 4-1BBL and, like murine 4-1BBL, is a type II glycoprotein with a single predicted transmembrane segment (22). Immobilized 4-1BBFc can induce monocytes to secrete cytokines, suggesting that human 4-1BBL may be involved in reverse sign- 

Thus, although human and murine 4-1BB and 4-1BBL have much in common, there are clearly differences. Furthermore, studies with human 4-1BBL to date have largely depended on the use of Abs and have examined the proliferation of unfractionated T cells (22, 36) or a Th1 clone (37). To further understand the role of 4-1BBL in human T cell responses, we have set up a model system to stimulate isolated human CD4 and CD8 T cells with 4-1BBL in conjunction with a TCR signal. As will be shown below, we find that indeed human CD4 and CD8 T cells can respond to 4-1BBL in the apparent absence of CD28 signaling and that 4-1BBL can augment both the expansion and the development of effector function of human T cell responses. The effects of 4-1BB on the CD8 T cell response are most apparent when both CD4 and CD8 T cells are present in the cultures; thus, CD4 and CD8 T cells cooperate in the response to 4-1BBL-mediated costimulation.

Materials and Methods

Cell lines and Abs

P815, EL4, and YAC-1 cells were obtained from American Type Culture Collection (Manassas, VA). The IL-2-dependent line CTL2-2 and the human monocyteic line THP-1 were also obtained from American Type Culture Collection. All cell lines were maintained in complete culture medium, which was prepared with RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FBS (Cansaera, Rexdale, Canada), 50 μM 2-ME, MEM nonessential amino acids (Life Technolo-
gies, Gaithersburg, MD), antibiotics, pyruvate, and glutamine.

The hybridomas OKT3, OKT4, and OKT8, secreting mAb specific for CD3, CD4, and CD8, respectively, were obtained from American Type Culture Collection. Abs were purified from hybridoma supernatants using protein G-Sepharose according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Piscataway, NJ) and conjugated to fluorescein using FITC (Molecular Probes, Eugene, OR), PE-conjugated anti-human 4-1BBL and anti-human CD3, and allophycocyanin-conjugated anti-IL-2 and anti-human IFN-γ were purchased from BD PharMingen (San Diego, CA). PE-conjugated as well as unconjugated anti-murine B7.1 (1G10) were also purchased from BD PharMingen. GL-1 (anti-B7.2) Ab (40) was isolated from hybridomas provided by Dr. R. Hodes (National Institutes of Health, Bethesda, MD) and purified on protein G-Sepharose. CTLA-4 Ig was purified as protein G separation from culture supernatants of CTLA-4 Ig-secreting cells (originally provided by Dr. P. Lane, University of Birmingham, Birmingham, U.K.). Anti-human CD8 Ab was isolated from hybridoma 9.3, provided by Dr. P. Poussier (University of Toronto, Toronto, Canada).

Generation of P815 cells expressing human 4-1BBL

Cyttoplasmic RNA for cDNA cloning was prepared from THP-1 monocytic cells using RNeasy mini kit (Qiagen, Mississauga, Canada). Human 4-1BBL cDNA was synthesized with the First Strand cDNA Syntheses kit (Roche, Indianapolis, IN) and PCR-amplified with HotStarTaq polymerase (Qiagen, Santa Clarita, CA). The transfection construct was made by insertion of the full-length human 4-1BBL cDNA into the EcoRI site of the pcDNA3 vector (Invitrogen, Carlsbad, CA).

P815 cells were transfected by electroporation with the human 4-1BBL construct and selected with Geneticin (Life Technologies, Grand Island, NY) for neomycin resistance. The resistant cells were sorted for human 4-1BB ligand expression with PE-conjugated anti-human 4-1BBL mAb, and a clone selected for high expression was used as a stimulator/target in the T cell functional assays. Cloned P815 cells were also transfected with pcdNA3 vector only (mock transfection), and a neomycin-resistant clone was used as a 4-1BBL negative control stimulator/target.

A previous report indicated that some tumor cells, including P815 cells, can express B7.1 after irradiation or treatment with cytotoxic drugs (41). Therefore, we analyzed B7.1 and B7.2 expression before and after irradiation of the P815-4-1BBL-expressing cells. Using 1G10 (anti-B7.1) and GL-1 (anti-B7.2) Ab, we found no detectable expression of B7.1 or B7.2 on P815 cells before or after irradiation under conditions where we could detect B7.1 expression on B7.1-expressing EL4 transfectants (data not shown).

t cell isolation

Human peripheral blood was collected from healthy volunteers after obtaining signed consent, as approved by the University of Toronto human subjects review board. More than 20 different donors were used for these studies, and each experiment was repeated with several different donors as indicated in the figures. Blood was mixed with an equal volume of PBS, overlaid on Ficoll-Paque Plus (Amersham Pharmacia, Oakville, Canada), and centrifuged at 300 × g for 20 min. Mononuclear cells were collected, washed twice with PBS, and resuspended in complete culture medium.

Cells in complete culture medium were incubated in culture flasks at 37°C for 1 h to deplete the plastic-adherent macrophages and monocytes. Non-adherent cells were collected in PBS and loaded onto isolation columns for total, CD45(+), or CD56+ human T cell separation (Cedarlane Laboratories, Hornby, Canada). T cells were eluted according to the manufacturer’s pro- tocol and resuspended in complete culture medium for functional assays.

T cell stimulation assays

Purified T cells were mixed with gamma-irradiated (80 Gy) stimulator cells at a 2:1 ratio (responder:stimulator) and cultured for 2–5 days. Culture supernatants were collected for cytokine assays, and analysis of CTL effector function. To prepare anti-CD3-loaded stimulatory cells, mock- or 4-1BBL-transfected P815 cells were suspended in PBS at 10^7 cells/ml, mixed with OKT3, and incubated at 37°C for 1 h, followed by three washes in PBS to remove unbound OKT3. Initial experiments involved titration of the anti-CD3 to determine the dose of OKT3 that when added to the stimulator cells gave minimal stimulation with OKT3 alone while revealing enhancement of T cell activation by 4-1BBL (data not shown). Based on these initial titrations, 0.25 μg OKT3 was added per 10^6 stimulator cells in all subsequent experiments. In some experiments anti-human CD8 or control Ab was also added to the P815 cells at the same time as OKT3 to test the effects of coligation of CD28 with OKT3 alone while revealing enhancement of T cell activation by 4-1BBL (data not shown). Based on these initial titrations, 0.25 μg OKT3 was added per 10^6 stimulator cells in all subsequent experiments. In some experiments anti-human CD8 or control Ab was also added to the P815 cells at the same time as OKT3 to test the effects of coligation of CD28 and OKT3 in the presence or the absence of 4-1BBL. In other studies we used CTLA-4 Ig or anti-murine B7.1 or B7.2 to test the effect of B7 on the stimulation. Blocking Abs were added to the cultures at the same time as T cells.

Analysis of cytokines in culture supernatants

IL-2 production was analyzed by measuring [^3H]thymidine incorporation of the IL-2-dependent cell line CTLL-2 after incubation with culture supernatants as described previously (10). IFN-γ levels were measured by ELISA using a cytokine detection kit (eBioscience, San Diego, CA) according to the manufacturer’s instructions.

Cytotoxic T cell assays

Cytotoxic T cell effector function was measured using a standard 51Cr release assay. Effector and target cells were cocultured at 37°C for 4 h, and the radioactivity of the supernatant was determined using a Top Count 96-well scintillation counter (Canberra-Packard, Meriden, CT).

Flow cytometry

All samples were analyzed on a FACS caliber (BD Biosciences, San Jose, CA) and analyzed by CellQuest software (BD Biosciences). For analysis of 4-1BB expression by flow cytometry, nonadherent, Ficoll-purified mononuclear cells from healthy volunteers were incubated with immobilized anti-CD3 Ab at a concentration of 1 μg/ml for the times indicated. At each time point the cells were stained with OKT4-FITC or OKT8-FITC, anti-CD3-CyChrome and 4-1BB-PE, or appropriate isotype control Abs. The cell populations were gated on CD3+ and CD4+ or CD8+ T cells.
For analysis of cytokine production by intracellular staining, samples were stimulated for 48 h, then treated for 6 h with Golgi-stop (BD Phar-Mingen) to inhibit cytokine release. Following surface staining for CD3 and CD4 or CD8, cells were fixed in Cytofix/Cytoperm solution (BD Phar-Mingen) and then stained with allophycocyanin-conjugated anti-human IFN-γ or anti-human IL-2 diluted in 1× perm/wash solution (BD PharMingen).

**Labeling of cells with fluorescent dyes**

T cells were stained with CFSE (Molecular Probes) as previously described (42). In brief, cells were resuspended in PBS at $5 \times 10^7 / \text{ml}$. CFSE was added to the cell suspension at a final concentration of 1 μM and incubated for 10 min at 37°C. Cells were washed three times in RPMI with 10% FCS and recounted.

**Results**

**Expression of 4-1BB on human CD4 and CD8 T cells**

A previous report indicated that 4-1BB is expressed on human CD4 and CD8 T cells, although the kinetics of this expression were not determined (17). To assess the kinetics of 4-1BB expression on CD4 vs CD8 T cells, adherent cell-depleted PBL from healthy donors were incubated with immobilized anti-CD3, and the expression of 4-1BB on CD4 vs CD8 CD3 cells was monitored by three-color flow cytometric analysis. Fig. 1 shows a representative example of one healthy donor. In total, we analyzed the kinetics of 4-1BB expression for 12 different donors. In the absence of stimulation there was no detectable expression of 4-1BB on the lymphocytes of all healthy donors examined. 4-1BB was detectable within 6 h of anti-CD3 stimulation and reached a maximum by ~48 h with most donors; where examined, 4-1BB expression was maintained at 72 h. In general, a greater proportion of CD8 T cells up-regulate 4-1BB and to higher levels than CD4 T cells. However, a proportion of both the CD4 and CD8 T cell subsets expresses 4-1BB after anti-CD3 stimulation in all individuals examined (Fig. 1 and data not shown).

**Expansion of T cells in response to 4-1BBL-mediated costimulation**

Having established that human CD4 and CD8 T cells can express 4-1BB rapidly upon anti-CD3 stimulation, we wished to assess the effect of 4-1BBL stimulation on these T cells. Full-length 4-1BBL cDNA or vector control was transfected into the P815 mastocytoma cell line. A subclone of the murine mastocytoma P815 was first isolated by limiting dilution to ensure that the parental cell line

**FIGURE 1.** Human 4-1BB is induced on peripheral blood lymphocytes from a healthy donor following stimulation with immobilized anti-CD3. Total T cells from 12 healthy donors were analyzed by three-color flow cytometry after staining with anti-CD3 CyChrome, anti-CD4- or anti-CD8-FITC, and anti-4-1BB-PE. The histograms shown are gated on CD3⁺ and CD4⁺ or CD8⁺, as indicated. The upper number indicates the percentage of cells positive for 4-1BB expression, and the lower number indicates the mean fluorescence intensity. Representative data are shown from a single donor.

**FIGURE 2.** Characterization of 4-1BBL transfected P815 cells. A, Expression of 4-1BBL on transfected P815 cells. Cloned P815 cells were transfected with human 4-1BBL or control vector (pcDNA3). Transfectants were stained with PE-conjugated anti-human 4-1BBL mAb and analyzed on a FACSCalibur. B, Binding of OKT3 to P815 or P815-H4-1BBL. FITC-labeled OKT3 was incubated with P815 cells as described in Materials and Methods. The solid line indicates fluorescence of cells incubated with OKT3-FITC, and the dotted line indicates cells not incubated with FITC-OKT3.
was homogeneous. A xenogeneic cell line was chosen to minimize any effects of endogenous costimulatory molecules. Fig. 2A shows flow cytometric analysis of P815 transfected with pcDNA3 vs P815 transfected with 4-1BBL. P815 cells express FcRs and can therefore be used to present anti-CD3 to T cells, thus providing a means of displaying the anti-TCR signal as well as the costimulatory signal on the same cell surface. Flow cytometric analysis was also used to determine that the mock-transfected P815 and 4-1BBL-transfected P815 bound similar levels of FITC-OKT3 (Fig. 2B).

To test the role of 4-1BBL in the expansion of total T cells vs CD4 or CD8 T cells, T cells were isolated as described in Materials and Methods and stimulated with 4-1BBL or vector-only-transfected P815 cells with or without anti-CD3 (OKT3 was added to P815 cells at 0.25 ng/10^6 cells, followed by washing). At the end of the 5-day culture the number of viable cells was determined by trypan blue exclusion, and the proportions of CD4 and CD8 T cells were analyzed by flow cytometry (Fig. 3). The isolated CD4 T cells expanded more than the isolated CD8 T cells (Fig. 3A). However, the relative proportions of CD4 and CD8 T cells in the unfractionated T cell cultures were not changed by stimulation (Fig. 3B). These results imply that both subsets have expanded to a similar extent in the cultures in which total T cells were stimulated with P815-4-1BBL plus OKT3. Similar results were found with four different donors. Based on the flow cytometric and cell counting data, both the CD4 and CD8 T cell populations increased by ~2-fold over the 5-day culture. This expansion reflects the net effect of survival and expansion vs cell death. These results indicate that 4-1BBL can stimulate the survival and/or expansion of both CD4 and CD8 T cells to a similar extent. Thus, the presence of CD4 T cells in the culture contributes to the CD8 T cell expansion. Fig. 3B also shows that only cultures that include anti-CD3 show evidence of blastogenesis of the T cells, as indicated in the forward vs side scatter profiles measured by flow cytometry. Furthermore, the proportion of enlarged cells is increased when cells are stimulated with P815-4-1BBL and OKT3 (Fig. 3B).

In contrast to the results with the P815 model shown here, attempts to stimulate T cells with 4-1BBL expressed on Chinese hamster ovary cells together with OKT3 in solution were not successful (data not shown), implying that it is essential to provide 4-1BBL and OKT3 on the same stimulatory surface.

To further assess the role of 4-1BBL in CD4 and CD8 T cell division, total T cells, isolated as described above, were labeled with CFSE and stimulated as shown in Fig. 3. After 1, 2, or 3 days of culture, the CFSE-labeled cells were surface stained with anti-CD4 or anti-CD8 and analyzed by flow cytometry (Fig. 4A). A decrease in the mean fluorescence intensity of the CFSE-labeled population, indicating that cell division had taken place, was observed in the cultures stimulated with both anti-CD3 and 4-1BBL. No division was observed in the first 24 h for three different donors (data not shown), and a very small proportion of cells had divided by 48 h. However, for all three donors examined (Fig. 4A and data not shown) both CD4 and CD8 T cells showed clear evidence of cell division by 72 h of culture, the CFSE-labeled cells were surface stained with anti-CD4 or anti-CD8 and analyzed by flow cytometry (Fig. 4A). A decrease in the mean fluorescence intensity of the CFSE-labeled population, indicating that cell division had taken place, was observed in the cultures stimulated with both anti-CD3 and 4-1BBL. No division was observed in the first 24 h for three different donors (data not shown), and a very small proportion of cells had divided by 48 h. However, for all three donors examined (Fig. 4A and data not shown) both CD4 and CD8 T cells showed clear evidence of cell division by 72 h of culture, indicating that cell division had taken place.

**FIGURE 3.** Analysis of T cell expansion in cultures stimulated with P815 cells expressing OKT3 and 4-1BBL. A, Purified total T cells or isolated CD4 T cells or CD8 T cells (1.3 × 10^6) were incubated with 0.7 × 10^6 irradiated (80 Gy) 4-1BBL or vector-only-transfected P815 stimulator cells with or without OKT3 in a 2-ml culture as indicated. After 5 days of culture, cells were recovered and viable cell numbers were assessed by trypan blue exclusion. Results are plotted as viable cell numbers observed per 10^6 input cells.

**FIGURE 4.** A, Analysis of cell division by CD4 and CD8 T cells responding to 4-1BBL-mediated costimulation

To assess the role of 4-1BBL in IL-2 production by T cells, unfractionated T cells or purified CD4 or CD8 T cells were incubated...
with P815 cells with or without 4-1BBL and with or without anti-CD3 as described above. Fig. 5A shows that in the presence of both 4-1BBL and anti-CD3 there was substantially more IL-2 production in cultures of total T cells or CD4 T cells than in cultures stimulated with anti-CD3 alone. In contrast, CD8 T cells produced only low levels of IL-2 in response to 4-1BBL-mediated costimulation. The observation that the CD8 T cell cultures contained little or no IL-2 in the supernatant following 4-1BBL-mediated costimulation provides an explanation for the poorer expansion of the isolated CD8 T cells in Fig. 3. However, in the total T cell cultures it appears that the CD4 T cells can produce sufficient IL-2 to allow similar expansion of both CD4 and CD8 T cell proliferation. The levels of cytokine measured in the supernatant of the cultures (Fig. 5A) reflect the net amount of IL-2 left in the supernatant after consumption by T cells in culture.

To determine the level of IL-2 produced by the CD4 and CD8 T cells in cultures of unfractionated T cells, we also examined IL-2 levels by intracellular staining and flow cytometry (Fig. 5B). A proportion of the CD4 T cells stimulated for intracellular IL-2 after 48 h of culture with P815 cells expressing 4-1BBL and anti-CD3, but no IL-2 was detectable in cells stimulated with anti-CD3 alone. As shown in Fig. 5A, CD8 T cells produced little or no IL-2 in response to anti-CD3 plus 4-1BBL stimulation and clearly produced less IL-2 than CD4 T cells.

We also analyzed the effects of anti-human CD28 on anti-CD3-mediated plus 4-1BBL-mediated IL-2 production. Anti-CD28 was used to coat the P815 cells at the same time as OKT3. To counter any effect of anti-CD28 on the binding of OKT3 to P815 cells, results were compared with those for P815 cells coated with OTK3 plus isotype control Abs. Fig. 5C shows...
FIGURE 5. Analysis of IL-2 production by T cells stimulated with 4-1BBL plus OKT3. A, Total T lymphocytes or isolated CD4 or CD8 T cells were stimulated as described in Fig. 3A, using vector-only-transfected or human 4-1BBL-transfected P815 cells, with or without OKT3, as indicated in the right panel. After 48 h, supernatants were removed and analyzed for IL-2 content using a bioassay with the IL-2-dependent cell line CTLL-2 as described in Materials and Methods. Results are reported as tritiated thymidine incorporation by CTLL-2 cells in response to dilutions of the culture supernatants. Data representative of four similar experiments. B, Samples stimulated as described in A were analyzed for IL-2 production by intracellular staining as described in Materials and Methods. The numbers on the plot represent the percentage of T cells in each quadrant. Qualitatively similar results were obtained using T cells from three different donors. C, In some experiments anti-CD28 or isotype control Ab (0.5 μg/ml/10^6 T cells) was added to the P815 cells together with anti-CD3. The presence of anti-CD28 enhanced the level of IL-2 production by the T cells responding to P815-4-1BBL for four different donors tested. D, Responses were blocked with 10 μg/ml CTLA-4Ig, a combination of anti-murine B7.1 and B7.2 (5 μg/ml each), or control Ab as indicated.
that coating of anti-CD28 on the 4-1BBL-expressing P815 cells enhances the amount of IL-2 produced over that induced by OKT3 plus P815-4-1BBL or by P815 plus OKT3, or anti-CD28 alone. Qualitatively similar results were obtained with four different donors. These data suggest that under conditions of limiting T cell stimulation, CD28 and 4-1BB signaling can both contribute to T cell activation.

Although we had determined that the P815 cells used in this assay did not express B7.1 or B7.2, it was conceivable that low levels of B7 family molecules present on the P815 cells, on activated T cells, or on contaminating APC contribute to the 4-1BBL-dependent response. To test this hypothesis, some experiments included CTLA-4Ig, a soluble inhibitor of B7-CD28 interaction at concentrations of up to 10 µg/ml. This concentration was sufficient to completely block an MLR against B7-expressing APC (30). As shown in Fig. 5D, CTLA-4Ig partially inhibited the response of human T cells to P815-4-1BBL plus OKT3 stimulation. In contrast, the combination of anti-murine B7.1 and anti-murine B7.2 in the same experiment showed no detectable blocking, implying that human B7 molecules either on activated T cells or on contaminating APC were enhancing the effects of human 4-1BBL. The magnitude of the effect of CTLA-4Ig on 4-1BBL-dependent T cell activation, compared with the marginal stimulation of human T cells in the absence of 4-1BBL, could be due to a synergistic effect of human 4-1BBL and human B7 family members in these cultures. However, the finding that even in the presence of high levels of CTLA-4Ig, there remains a significant 4-1BBL-induced response indicates that 4-1BBL can induce some CD28-independent activation of human T cells.

4-1BBL augments IFN-γ production by CD4 and CD8 T cells

Fig. 6A shows the time course of IFN-γ production in cultures of purified T cells incubated with irradiated P815 cells with or without 4-1BBL and with or without OKT3. The combination of 4-1BBL and anti-CD3 on P815 cells allows IFN-γ production by day 2 of culture, whereas 4-1BBL or anti-CD3 alone does not support IFN-γ production by T cells. Thus, 4-1BBL can provide a costimulatory signal for IFN-γ production by purified human T cells. This finding is consistent with the previous results reported by Kim et al. (37), who observed that anti-4-1BB could enhance Th1 cytokine production by a Th1 clone responding to anti-CD3 plus anti-CD28. To determine which population of T cells, CD4 or CD8, was making IFN-γ in the cultures, samples were also analyzed for intracellular cytokine staining by the CD4 and CD8 T cell subsets (Fig. 6B). After 48 h of culture with P815-4-1BBL plus anti-CD3, a small proportion of both CD4 and CD8 T cells was positive for IFN-γ production. Negligible numbers of IFN-γ-producing T cells were found in cultures stimulated with only P815 plus anti-CD3 or with P815-4-1BBL in the absence of anti-CD3 (Fig. 6B). The donor in Fig. 6B showed a higher proportion of CD4 compared with CD8 T cells producing IFN-γ. However, other donors showed the reverse, with a higher proportion of CD8 T cells producing IFN-γ. However, all three donors tested consistently showed that P815-4-1BBL together with anti-CD3 could induce IFN-γ production by both T cell subsets.

Role of 4-1BBL in augmentation of CTL effector function

4-1BBL has been shown to augment the development of CTL effector function by mouse CD8 T cells. To determine the effect of 4-1BBL on the development of human cytolytic T cell activity, purified T cells were incubated with P815 cells with and without anti-CD3 and 4-1BBL as described above. After 5 days of culture, T cells were tested for their ability to kill 51Cr-labeled P815 cells. The presence of both 4-1BBL and OKT3 on the stimulator cells resulted in a substantial increase in CTL activity against the P815 targets over stimulation with
P815-OKT3 or P815-4-1BBL alone. However, 4-1BBL on P815 cells also showed a small anti-CD3-independent effect on the development of CTL effector function (Fig. 7A). Once stimulated with 4-1BBL and anti-CD3, the effector CTL could kill the P815 targets regardless of the presence of anti-CD3 or 4-1BBL (Fig. 7A). Thus, the CTL appear to have developed a specific (presumably MHC-restricted) response against P815 cells during the 5-day culture, and the development of this response was augmented by 4-1BBL and even more so when 4-1BBL was provided together with OKT3.

To further substantiate the conclusion that T cells have developed a specific response to P815 cells, CTL effector cells from P815-stimulated cultures were tested for the ability to kill other targets (Fig. 7B). T cells that had been stimulated with P815-4-1BBL plus OKT3 were able to kill P815 (H-2d) cells and, to a lesser extent, another H-2d target (A20), but 4-1BBL stimulation had a negligible effect on subsequent killing of an MHC-unrelated target, EL4 (H-2b), or the mouse NK-sensitive target, YAC-1 (Fig. 7B). These results suggest that the cytolytic response that has developed is specific to the stimulating cells and is dependent on the presence of anti-CD3 during the initial stimulation, but not during the killing. The response is also augmented by the presence of anti-CD3 during the initial culture. The response of T cells to 4-1BBL requires that T cells receive a signal through the TCR to up-regulate 4-1BB. The presence of anti-CD3 in the cultures is expected to result in more effective up-regulation of 4-1BB and may enhance the ability of 4-1BBL to augment the development of the CTL response to P815 target cells.

Discussion

The data presented in this report indicate that human 4-1BBL can provide a costimulatory signal for human T cell activation, thereby allowing T cell expansion as well as cytokine production and the development of CTL effector function. Thus, as previously demonstrated for murine 4-1BBL, human 4-1BBL can function as a costimulatory molecule for both CD4 and CD8 T cell activation. For murine T cells we recently reported that isolated CD4 and CD8 T cells expand to a similar extent in response to anti-CD3 plus 4-1BBL-mediated costimulation (13). In the present study when total T cells were stimulated with 4-1BBL together with anti-CD3 on the surface of P815 cells, both CD4 and CD8 T cells expanded to a similar extent, consistent with both CD4 and CD8 T cells responding to 4-1BBL-mediated costimulation. This result was observed both by determining the number of CD4 and CD8 T cells at the end of the 5-day culture and by analyzing cell division over the first 3 days of culture using CFSE labeling (Figs. 3 and 4). In contrast to the results with unfractionated T cells, in cultures of isolated CD8 T cells the amount of T cell expansion was less (Fig. 3); this is attributed to the lower levels of IL-2 produced by human CD8 T cells compared with CD4 T cells (Fig. 4). These studies demonstrate that for human T cells, 4-1BBL exerts its maximal effect in cultures containing both CD4 and CD8 T cells. The expansion of T cells in P815/OKT3/4-1BBL-stimulated cultures was relatively modest (~2-fold). The recovery of cells in the cultures reflects the net effects of cell death vs cell survival and/or division.

In these cultures there were no detectable B7 family members on the stimulator cells, so the only source of B7 family members would be on contaminating APC or activated T cells. The response of human T cells to OKT3 plus 4-1BBL stimulation was partially blocked by addition of CTLA-4Ig, suggesting that some B7 family members present in the culture, probably on activated T cells or contaminating APC, contribute to the T cell activation. Alternatively, CTLA-4Ig binding to B7.2 on activated T cells might also block the stimulation due to steric hindrance. The finding that vector-only-transfected P815 cells give minimal stimulation in the
presence of OKT3 in these cultures, whereas 4-1BBL-transfected P815 cells stimulate with OKT3 in a partially B7-dependent manner, could be due to a synergy between the B7 and 4-1BBL costimulatory pathways. Consistent with this finding, addition of anti-CD28 to the cultures clearly augmented IL-2 production (Fig. 5C). Additionally, 4-1BBL itself might contribute to up-regulation of B7.2 expression on T cells. Nevertheless, even in the presence of a high dose of CTLA-4Ig in the cultures, 4-1BBL was able to stimulate a response in T cells. Thus, the results presented here suggest that, like murine 4-1BBL, human 4-1BBL can promote CD28-independent T cell activation, but the combination of CD28- and 4-1BB-mediated costimulation is more effective than either alone.

4-1BBL also augmented the development of CTL effector function (Fig. 7). Once CTL activity was induced, however, the presence of 4-1BBL on target cells did not effect the level of lysis. These data imply a role for 4-1BBL in the expansion of CTL with concomitant development of CTL effector function, but rule out a role for 4-1BB/4-1BBL in the actual killing function of CTL. Similar findings were observed with murine 4-1BBL (14). Although the generation of maximal CTL activity in the cultures required both OKT3 and 4-1BBL on P815 stimulator cells, 4-1BBL on P815 alone induced some activity, consistent with the finding that we had induced a specific MHC-restricted response to P815 cells that becomes independent of anti-CD3 in the cultures by the time of the 51Cr release assay. The enhancement of development of CTL activity due to the presence of anti-CD3 may be due in part to the requirement for a strong signal through the TCR to induce 4-1BB expression, a prerequisite for the response to 4-1BBL.

Kinetic analysis of 4-1BBL expression indicated that CD8 T cells up-regulated 4-1BB to a greater extent and more rapidly than CD4 T cells when unfractonated lymphocyte cultures were stimulated with plate-bound anti-CD3. Despite the differences in the level of 4-1BB up-regulation by CD4 vs CD8 T cells, the studies in Fig. 3 imply that both CD4 and CD8 T cells respond similarly to 4-1BBL-mediated costimulation in terms of net expansion observed in the cultures.

With respect to cytokine production, CD4 T cells were more responsive to anti-CD3 plus 4-1BBL in IL-2 production, and both CD4 and CD8 T cells produced IFN-γ in response to 4-1BBL/anti-CD3 stimulation. The findings that human 4-1BBL stimulates IL-2 production by CD4 T cells and IFN-γ production by CD8 T cells are similar to the results obtained with mouse 4-1BBL (13). We did not observe IL-4 production by either ELISA or intracellular cytokine staining in these studies (data not shown). However, we have not attempted to drive Th2 cytokine production in this model. In contrast, mouse 4-1BBL expressed on a B lymphoma leads to IL-2 and IL-4 production by CD4 T cells, and IFN-γ production as well as low levels of IL-2 production by CD8 T cells (10, 13). The murine CD4 T cell response to 4-1BBL can be switched to a Th1 response if IL-12 is added to the cultures (10). Thus, it seems likely that other factors besides 4-1BBL predominate in Th1/Th2 commitment by the T cells. Rather, 4-1BBL seems to function as a more general stimulus for T cell activation.

Taken together our data suggest that 4-1BBL can be used to augment the activation of human CD4 and CD8 T cells, and that 4-1BBL in conjunction with anti-CD3 provides an effective method for expanding CD4 and CD8 T cells with effector function. For CD8 T cells this expansion is most efficient when both CD4 and CD8 T cells are present in the same culture.


