

## 4-1BB Costimulatory Signals Preferentially Induce CD8<sup>+</sup> T Cell Proliferation and Lead to the Amplification In Vivo of Cytotoxic T Cell Responses

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### Summary

The 4-1BB receptor is an inducible type I membrane protein and member of the tumor necrosis factor receptor (TNFR) superfamily that is rapidly expressed on the surface of CD4<sup>+</sup> and CD8<sup>+</sup> T cells after antigen- or mitogen-induced activation. Cross-linking of 4-1BB and the T cell receptor (TCR) on activated T cells has been shown to deliver a costimulatory signal to T cells. Here, we expand upon previously published studies by demonstrating that CD8<sup>+</sup> T cells when compared with CD4<sup>+</sup> T cells are preferentially responsive to both early activation events and proliferative signals provided via the TCR and 4-1BB. In comparison, CD28-mediated costimulatory signals appear to function in a reciprocal manner to those induced through 4-1BB costimulation. In vivo examination of the effects of anti-4-1BB monoclonal antibodies (mAbs) on antigen-induced T cell activation have shown that the administration of epitope-specific anti-4-1BB mAbs amplified the generation of H-2<sup>d</sup>-specific cytotoxic T cells in a murine model of acute graft versus host disease (GVHD) and enhanced the rapidity of cardiac allograft or skin transplant rejection in mice. Cytokine analysis of in vitro activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells revealed that anti-4-1BB costimulation markedly enhanced interferon- $\gamma$  production by CD8<sup>+</sup> T cells and that anti-4-1BB mediated proliferation of CD8<sup>+</sup> T cells appears to be IL-2 independent. The results of these studies suggest that regulatory signals delivered by the 4-1BB receptor play an important role in the regulation of cytotoxic T cells in cellular immune responses to antigen.

**4**-1BB is a recently cloned pan T cell activation antigen and member of the low affinity nerve growth factor receptor/tumor necrosis factor receptor (NGFR/TNFR)<sup>1</sup> family of integral type I membrane proteins (1). In addition to the NGFR, the two TNFR and 4-1BB, other members of this family include CD27, CD30, CD40, OX-40, and Fas (2). Similar to other members of this family, 4-1BB can provide either costimulatory signals leading to activation, IL-2 production and enhanced proliferation and differentiation, or death signals to T cells, depending on the state of activation and stage of differentiation of the T cell (3–5). The biochemical pathways utilized by the 4-1BB receptor for conveying these activation signals have not yet been

elucidated. However, the cytoplasmic domain of murine 4-1BB contains the consensus sequence (Cys-X-Cys-Pro) for binding p56<sup>Lck</sup> and it has been shown that Lck can be immunoprecipitated using mAbs to the 4-1BB receptor (1). This observation suggests that 4-1BB may be linked to the *src* tyrosine kinase family signaling pathway. Although not expressed in resting T cells, 4-1BB mRNA can be detected within 1.5 h of CD3/TCR or PMA/ionomycin stimulation (6), followed by cell surface expression within 10 h of activation (our personal observation). Recently, a high affinity ligand for 4-1BB (4-1BBL) was cloned and the protein was shown to be a type II membrane protein of the TNF family (7). Thus, like all of the other members of the TNFR family, the ligand for 4-1BB is also a member of the TNF family of molecules. 4-1BBL is expressed in low levels on resting B cells and upregulated on activated B cells and monocytes (7). Using 4-1BB fusion proteins or 4-1BB transfected cell lines it has been shown that engagement of

<sup>1</sup>Abbreviations used in this paper: 4-1BBL, 4-1BB ligand; EU, endotoxin units; FBS, fetal bovine serum; NGFR, nerve growth factor receptor; SN, activated T cell supernatants.

4-1BBL on B cells provides them with an anti- $\mu$ -dependent costimulatory signal that amplifies B cell proliferation (8). In addition to the high affinity ligand for 4-1BB, we have found that murine 4-1BB binds to extracellular matrix proteins (9). The biological significance of this observation is presently under investigation.

Currently, all published studies addressing the functional activities of the 4-1BB/4-1BBL system have been carried out in vitro and have focused on costimulatory signals generated through the 4-1BB receptor on unseparated T cells. In this study, we show that anti-4-1BB mAbs preferentially activate CD8<sup>+</sup> T cells both in vitro and in vivo, alter the kinetics of tyrosine phosphorylation as well as substrates and markedly amplify the generation of antigen-specific CTL responses in vivo. In this regard, costimulation through 4-1BB and CD28 are reciprocal in nature and complimentary to one another by activating individually CD8<sup>+</sup> and CD4<sup>+</sup> T cells, respectively. The results of our investigation also suggest that anti-4-1BB mAbs and mAb derivatives may have novel immunoregulatory properties that will prove useful for the development of clinical reagents for the treatment of chronic infectious diseases and cancer.

## Materials and Methods

**Construction of 4-1BB Fusion Proteins.** The murine 4-1BB-Ig fusion construct was generated in the following manner: DNA encoding the extracellular domain of murine 4-1BB (residues 22–185) was generated by PCR using an upstream primer containing a KpnI site (5'-CCGCGGGTACCCGTGCAGAACTCCTGTGATAA-CTGT-3') and a downstream primer containing a BamHI site (5'-CCGCTACGTAGGATCCTGCAAGGAGTGCCCTCC-TGG-3'). The murine 4-1BB DNA fragment was cloned in frame into the CDM7(B<sup>-</sup>) vector that contains the CD5 signal sequence, followed by a KpnI site and the human immunoglobulin constant region preceded by a BamHI site.

The murine 4-1BBL-CD8 fusion construct was prepared as follows: DNA encoding the extracellular domain of murine 4-1BBL (residues 104–309) was generated by PCR using an upstream primer containing a BamHI site (5'-GCGGCGGATCCCCGC-ACCGAGCCTCGGCCAGCG-3') and a downstream primer containing an XbaI site (5'-CGCTCTAGAGGATAGTTCTCA-TTCCCATGG-3'). The murine 4-1BBL DNA fragment was cloned in frame into the CDM7(B<sup>-</sup>) vector containing the extracellular domain of CD8 followed by a BamHI site.

**Generation and Characterization of mAbs.** Monoclonal anti-mouse 4-1BB mAbs were generated by immunizing Lewis rats with 4-1BB fusion proteins prepared as described earlier (10). Using immune spleen cells and the mouse myeloma X63-AG8.653 cell line as fusion partner (11), rat-mouse hybridomas were generated and selected using standard published techniques.

**Biacore Analysis.** All studies were carried out on a Biacore instrument equipped with the Biacore 1000 upgrade kit (Pharmacia Biosensor, Uppsala, Sweden). Experiments were carried out at 25°C using PBS, pH 7.4, containing 0.0005% surfactant P20 (Pharmacia Biosensor) as the running buffer. The carboxymethylated dextran matrix of research grade sensor chip CM5 was modified using the amine coupling kit as follows: equal volumes of 0.10 M NH<sub>2</sub>-hydroxysuccinimide and 0.40 M *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide were mixed and injected to activate

the surface for 4 min. The receptor, a solution of 4-1BB-IgG in 10 mM sodium formate, pH 4.0, was injected for 4 min. Remaining active sites were reacted by the injection of 1 M ethanolamine for 5 min. Immobilization of ~1,000 RU of receptor was achieved. Each of the antibodies and fusion protein was diluted to 67 nM in the running buffer and 100  $\mu$ l was injected at a flow rate of 5  $\mu$ l/min, followed by running buffer alone to allow observation of the dissociation of bound protein. After each cycle, the surface was regenerated by the injection of 1 M formic acid, 0.15 M NaCl for 30 s. Association and dissociation rates for each sensorgram were determined by curve fitting using BIAevaluation 2.1 software. The affinity was calculated by dividing the dissociation rate by the association rate. The association rates for two antibodies, 1D8 and 21E5, were confirmed by the method of doing a series of injections at concentrations ranging from 10 to 100 nM. BIAevaluation 2.1 was used to fit the data to the appropriate model.

**Purification of Proteins.** The 3B8 mAb (IgM) was affinity purified on an anti- $\kappa$  chain (mAb RG7) column. All other mAbs (IgG2a) were purified on protein G (Gammabind Plus; Pharmacia). All antibodies were eluted with IgG elution buffer (Pierce Chem. Co., Rockford, IL), neutralized immediately, dialyzed versus PBS, and filter sterilized. Endotoxin levels in the purified antibodies were less than 0.3 EU/mg, except for the 21E5 mAb (1.2 EU/mg) and 5C12 (0.5 EU/mg). The negative control mAb, 6E9, was a rat IgG2a antibody, reactive with human gp-39. The 4-1BBL-CD8 (Lyt2a) fusion protein was affinity purified using an anti-CD8 column (mAb 53.6). The protein was eluted with 40% propylene glycol, 60% 50 mM Tris, pH 7, with 1.25 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>.

**T Cell Activation and FACS<sup>®</sup> Analysis.** The D0-11-10 T cell hybridoma was treated with 10 ng/ml PMA and 0.5  $\mu$ g/ml ionomycin. The cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin solution (GIBCO BRL, Gaithersburg, MD). The cells were subsequently phenotyped by indirect immunofluorescence and FACS<sup>®</sup> analysis using a FACStar<sup>®</sup> flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA) for 4-1BB expression. D0.11.10 cells express 4-1BB on the cell surface within 10 h of stimulation and expression peaks between 12–14 h. To evaluate the ability of the various anti-4-1BB mAbs to block ligand receptor binding, activated D0-11-10 were incubated at 4°C, with 0.02% azide for 1 h with various concentrations of the anti-4-1BB mAbs then purified 4-1BBL-CD8 fusion protein was added to a final concentration of 1  $\mu$ g/ml and the incubation continued for 1 h. Cells were then washed and ligand binding determined with phycoerythrin-conjugated 53.6 (anti-CD8) (Boehringer Mannheim).

**Splenic T Cell Activation for Induction of 4-1BB Expression.** BALB/c splenic T cells were purified from unseparated cells by treatment with anti-IA<sup>d</sup> mAb (PharMingen) and anti-CD11b (PharMingen) at 12  $\mu$ g/ml each per  $1 \times 10^8$  spleen cells/ml followed by incubation at 4°C for 20 min. The cells were washed with PBS (2  $\times$  50 ml) and resuspended at 2–4  $\times 10^8$ /ml, followed by the addition of 0.4 ml of magnetic beads coated with goat anti-mouse IgG (Dyna) 2 ml of cell suspension. The cell suspension was then incubated at 4°C on a rotator for 10 min, after which the cell volume was increased to 8 ml with PBS. Non-T cells bound to the magnetic beads were removed using a magnetic field. The remaining cells were washed, resuspended to 2 ml, and again placed in a magnetic field to remove residual beads. Washed T cells were then resuspended to  $1 \times 10^7$ /ml in RPMI 1640 containing antibiotics and 10% FBS and phenotyped by FACS<sup>®</sup> for CD3 expression and found to be >95% CD3<sup>+</sup>. T cells were activated for varying periods of time up to 72 h by

adding hamster anti-CD3 (145.2C11, PharMingen) at 1  $\mu\text{g}/\text{ml}$  together with 0.5% non-T cells to the cultures and incubating at 37°C. In some cases, splenocytes were first activated and then the non-T cells were removed as described above before using the activated T cells.

**T Cell Proliferation Assays.** BALB/c splenic T cells were prepared as described earlier. CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets were prepared by negative selection using anti-CD4 and anti-CD8 mAbs (PharMingen) and magnetic beads (Dyna). The protocol followed was that supplied by the manufacturer. Unseparated T cells or T cell subsets were cultured in 96-well flat-bottomed microtiter plates at a density of  $1 \times 10^5/\text{well}$  in 200  $\mu\text{l}$  of RPMI 1640 containing 10% FBS and 1% l-glutamine and penicillin/streptomycin (GIBCO BRL). In addition, cultures were supplemented with 0.5% non-T cells as a source of FcR<sup>+</sup> cells used to present mAbs to the T cells. Cultures were maintained at 37°C for the indicated periods. During the final 12 h of culture the cells were pulsed with 1  $\mu\text{Ci}/\text{well}$  [<sup>3</sup>H]thymidine (NEN, Boston, MA). All cultures were harvested and counted by liquid scintillation spectroscopy.

**GVHD Experiments and CTL Assay.** GVHD was established in 8- to 10-wk-old BDF<sub>1</sub> (H-2<sup>bd</sup>) female mice by tail vein injection of  $1 \times 10^7$  purified C57BL/6 (H-2<sup>b</sup>) T cells. Recipient mice received at the time of T cell injection and again on days 2 and 4, 200  $\mu\text{g}$  of anti-4-1BB mAb, an equivalent amount of isotype-matched negative control mAb (6E9), which is reactive with human gp-39, or PBS. On day 10, mice were killed and splenic single cell suspensions prepared, and  $2 \times 10^4$  <sup>51</sup>Cr-labeled P815 cells cultured in 200  $\mu\text{l}$  of RPMI 1640 containing 10% FBS in 96-well round-bottomed microtiter plates. In some instances, T cells were first isolated and used as effectors in CTL assays. The plates were spun at 1,000 rpm for 1 min before incubating at 37°C for 4 h. After the incubation period, 100  $\mu\text{l}$  of culture supernatant was collected and counted in triplicate in a Packard Cobra  $\gamma$  counter. Specific <sup>51</sup>Cr-release was calculated in percent using the formula:  $\text{sample release} - \text{spontaneous release} / \text{total release} - \text{spontaneous release} \times 100$ .

**Western Blots.** Cell lysates were prepared from 48-h anti-CD3-activated BALB/c splenic T cells that had been fractionated into CD4<sup>+</sup> and CD8<sup>+</sup> subsets after activation. Both subsets were then restimulated with anti-CD3, anti-4-1BB, or a combination of the two for the times indicated. The cells were then lysed in 1% NP-40 lysis buffer containing protease and phosphatase inhibitors. Lysates containing  $1 \times 10^7$  cells/ml were immunoprecipitated overnight using 5  $\mu\text{l}$  of rabbit anti-phosphotyrosine antibody (Zymed Labs, San Francisco, CA) and 25  $\mu\text{l}$  of protein G-Sepharose beads (Pharmacia-Biotech, Uppsala, Sweden) at 4°C. After extensive washing of the beads, 50  $\mu\text{l}$  of 2 $\times$  sample buffer were added and the mixture was heated to 100°C for 5 min. The samples were then loaded on 4–20% density gradient gels and electrophoresed. Gels were then electroblotted at 20 V overnight, blocked with 6% BSA/PBS, and blotted with a 1:5,000 dilution of AP-conjugated monoclonal anti-phosphotyrosine antibody 4G10 (UBI, Lake Saranac, NY) overnight at 4°C. Blots were washed three times in PBS for 5'/wash, one time in PBS-.002% Tween 20, and one time in PBS before adding ECL reagents for 1 min, followed by exposure to film.

**Anti-4-1BB-induced Cytokine Production.** Resting CD4<sup>+</sup> and CD8<sup>+</sup> BALB/c splenic T cells ( $1 \times 10^6/\text{ml}$ ) were stimulated for the indicated periods with anti-CD3 at 1  $\mu\text{g}/\text{ml}$  with or without 10  $\mu\text{g}/\text{ml}$  anti-4-1BB (3H3) or anti-CD28 (3N7). Culture supernatants were collected and assayed by ELISA assay for IL-2, IL-4, IL-10, and IFN- $\gamma$ .

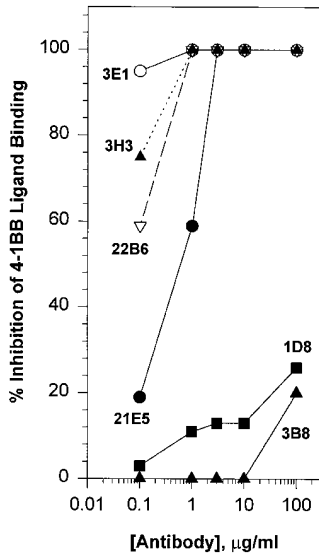
**Table 1.** Plasmon Resonance Analysis of Anti-4-1BB mAb Binding to 4-1BB-Ig Fusion Protein

Antibody	Isotype	$k_a$	$k_d$	$k_d/k_a$
		$M^{-1}s^{-1}$	$s^{-1}$	$M$
1D8	IgG <sub>2A</sub>	$2.9 \times 10^5$	$1.2 \times 10^{-4}$	$4.14 \times 10^{-10}$
22B6	IgG <sub>2A</sub>	$5.7 \times 10^5$	$1.5 \times 10^{-4}$	$2.63 \times 10^{-10}$
3H3	IgG <sub>2A</sub>	$3.7 \times 10^5$	$1.9 \times 10^{-4}$	$5.14 \times 10^{-10}$
15B9	IgG <sub>2A</sub>	$4.3 \times 10^5$	$1.1 \times 10^{-4}$	$2.56 \times 10^{-10}$
3E1	IgG <sub>2A</sub>	$5.7 \times 10^5$	$1.3 \times 10^{-5}$	$2.28 \times 10^{-11}$
5C12	IgG <sub>2A</sub>	$5.6 \times 10^5$	$1.5 \times 10^{-4}$	$2.68 \times 10^{-10}$
21E5	IgG <sub>2A</sub>	$5.3 \times 10^5$	$1.1 \times 10^{-4}$	$1.99 \times 10^{-10}$
3B8	IgM	$2.8 \times 10^{-5}$		
4-1BB Ligand		$1.83 \times 10^5$	$2.13 \times 10^{-4}$	$1.16 \times 10^{-9}$

## Results

**Generation of Anti-4-1BB mAbs.** Using the published sequences for murine 4-1BB and 4-1BBL, we constructed fusion proteins containing the extracellular domain of 4-1BB and the COOH region of human IgG<sub>1</sub> (9). 4-1BBL fusion protein was constructed using the 4-1BBL extracellular domain fused with the NH<sub>2</sub>-terminal region of murine CD8 (data not shown). 4-1BB-Ig fusion protein was used to immunize rats in order to generate mAbs. More than a dozen hybridomas having unique specificity for 4-1BB were selected on the basis of ELISA binding assay on purified 4-1BB-Ig fusion protein, activated T cell reactivity, and immunoprecipitation of a 36-kD protein (data not shown). All of the mAbs were of the rat IgG<sub>2A</sub> isotype except for 3B8, which was found to be a rat IgM antibody. These mAbs were then analyzed by surface plasmon resonance in order to determine their association and dissociation rates as well as their avidity for antigen (Table 1). We also determined the ability of all mAbs to block 4-1BB/4-1BBL binding on activated, 4-1BB<sup>+</sup>, D0.11.10 cells. The results for several of these mAbs is shown in Fig. 1. From this experiment, it can be seen that there is considerable heterogeneity among the tested antibodies with respect to their ability to block 4-1BBL binding. Through 4-1BB domain swapping experiments we found that the 1D8 binding site was located near the NH<sub>2</sub>-terminal end of the protein and away from the ligand binding region of the molecule (data not shown). All of the remaining antibodies bind at or near the ligand binding site, including the IgM isotype mAb 3B8. However, the low binding avidity of 3B8 presumably prevents it from effectively blocking ligand binding to 4-1BB.

**Costimulatory Properties of Anti-4-1BB Mabs.** All of the anti-4-1BB were tested for their ability to costimulate T cell proliferation induced by the 145.2C11 hamster anti-mouse CD3 mAb. Anti-4-1BB mAbs at 10  $\mu\text{g}/\text{ml}$  were added to T cell cultures in which anti-CD3 mAb was titrated from 3–100 ng/ml. In Fig. 2, the results of a typical experiment are shown for several selected mAbs. At a con-

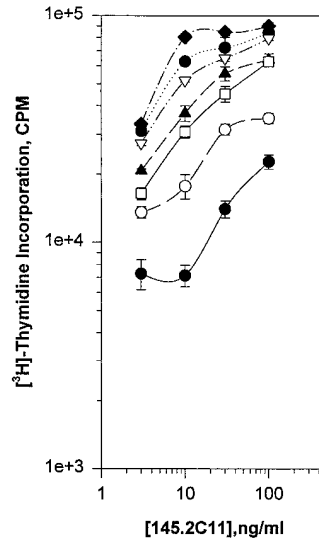


**Figure 1.** Anti-4-1BB mAbs block 4-1BBL binding to activated DO.11.10 T cells. T cells were activated as described above. Cells were then incubated with dilutions of anti-4-1BB mAbs for 30 min before the addition of 4-1BBL-CD8 fusion protein at 1  $\mu\text{g}/\text{ml}$  and the cells were incubated for an additional 30 min. Cells were washed twice in PBS, incubated with PE-conjugated anti-CD8 mAb, and assayed by FACS<sup>®</sup> for ligand binding.

centration of 3 ng/ml of anti-CD3, anti-4-1BB mAbs enhanced the proliferative response from two- to eightfold depending upon the specific mAb used. Anti-4-1BB mAbs in the absence of anti-CD3 had no stimulatory effect on proliferation of T cells (data not shown).

**Costimulation of CD4<sup>+</sup> and CD8<sup>+</sup> T Cells via 4-1BB Receptor.** 4-1BB expression on activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells occurs with the same kinetics and degree of expression (data not shown). Although both subsets express 4-1BB at equivalent levels it is not known whether the 4-1BB receptor preferentially or differentially regulates the activation or proliferative potential of either subset. To test this possibility, we isolated highly purified populations of resting splenic BALB/c CD4<sup>+</sup> or CD8<sup>+</sup> T cells (>98% purity) by negative selection procedures described earlier. T cell subsets were then incubated with soluble 145.2C11 mAb in a range of 10–1,000 ng/ml  $\pm$  10  $\mu\text{g}/\text{ml}$  anti-4-1BB mAb for 3 d in the presence of 0.5% APC. Proliferation was monitored by [<sup>3</sup>H]thymidine incorporation during the final 12 h of culture. In Fig. 3 A we show the results of one of four proliferation experiments carried out on isolated CD4<sup>+</sup> T cells or CD8<sup>+</sup> T cells obtained from the same group of mice. From these experiments, it is clear that costimulation of CD4<sup>+</sup> T cells with anti-4-1BB mAbs such as 3E1 and 3H3 led to a two- to threefold enhancement of anti-CD3-induced proliferation, whereas others such as 1D8, a nonligand-blocking antibody, had little effect upon T cell proliferation. mAb 6E9 is an isotype-matched negative control that is reactive with human gp-39. In contrast, the same set of antibodies markedly enhanced CD8<sup>+</sup> T cell proliferative responses by lowering the threshold of anti-CD3 concentration 10- (1D8) to 100-fold (3H3) and increased the incorporation of [<sup>3</sup>H]thymidine  $\sim$ 100-fold.

**4-1BB Initiates Proliferative Signals to T Cells.** Because the most efficient anti-4-1BB antibodies capable of inducing T cell proliferation happen to be those that effectively block ligand-receptor binding, we questioned whether or not antibody-mediated proliferation was initiated by the con-

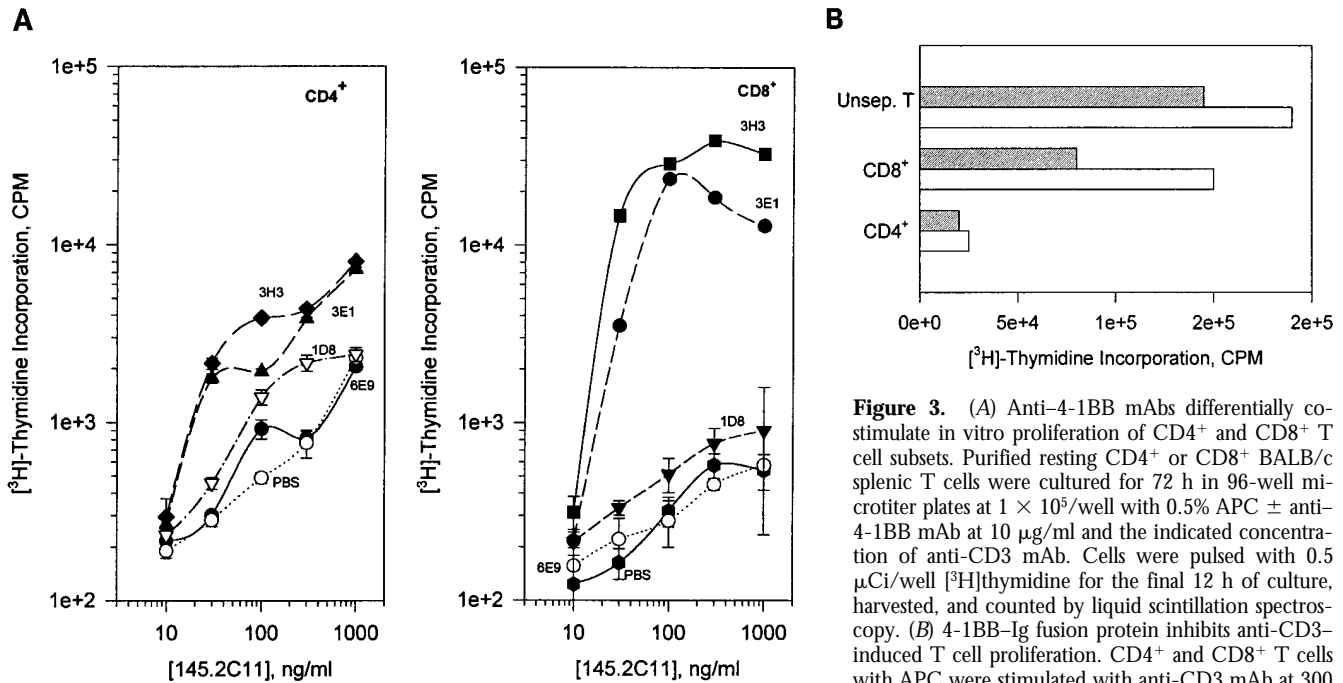


**Figure 2.** Anti-4-1BB mAbs costimulate anti-CD3-induced T cell proliferation. BALB/c T cells were cultured for 72 h at  $2 \times 10^5/\text{well}$  in 96-well flat-bottomed microtiter plates with the indicated concentrations of 145.2C11 with PBS control (●) or with the following: anti-4-1BB mAbs 15B9 (□), 1D8 (○), 21E5 (▲), 3B8 (●), 3H3 (◆), and 3E1 (▽), each at 10  $\mu\text{g}/\text{ml}$ . Results are expressed as the mean  $\pm$  SD of triplicate wells.

veyance of a positive signal to the T cell, or inhibition of a negative signal provided by 4-1BBL binding to its receptor. To address this question, we stimulated T cell subsets as well as unseparated T cells in the presence of APCs (5%) with anti-CD3 mAb with or without soluble 4-1BB-Ig fusion protein. As shown in Fig. 3 B, the inclusion of 4-1BBL into all T cell cultures diminished the proliferative responses initiated by anti-CD3 stimulation. Also of note was the observation that CD8<sup>+</sup> T cells were the most adversely affected by this treatment. These results support our findings that anti-4-1BB mAbs preferentially activate CD8<sup>+</sup> T cells.

**CD28 and 4-1BB Preferentially Costimulate Reciprocal T Cell Subsets.** As a result of our observation that 4-1BB costimulation is markedly more effective in CD8<sup>+</sup> T cells than CD4<sup>+</sup> T cells, we compared these findings to those obtained after costimulation of each subset with anti-CD28 (3N7) mAbs. In Fig. 4 we show a typical experiment in which the kinetics of costimulation were studied on CD4<sup>+</sup> T cells or CD8<sup>+</sup> T cells using antibodies to either 4-1BB (3H3 and 3E1) or CD28 (3N7). The results of this experiment clearly demonstrate that 4-1BB and CD28 molecules preferentially activate CD8<sup>+</sup> and CD4<sup>+</sup> T cell subsets, respectively, and that 4-1BB costimulation peaks at 48 h.

**Induction of Protein Tyrosine Phosphorylation by Anti-4-1BB mAb.** In light of the observed selective effect that anti-4-1BB mAbs had on CD8<sup>+</sup> T cell proliferation, we analyzed the ability to 3E1 (or 3H3; data not shown) to influence early signal transduction events in purified populations of 48-h anti-CD3-activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells. After a 15-min incubation with 3E1, 145.2C11, or both (each mAb at 10 ng/ml) at room temperature, the cells were placed in a water bath equilibrated to 37°C and incubated for the indicated times after the addition of 50 ng/ml of a mouse anti-rat mAb, RG7, that cross-reacts with hamster mAb. When both anti-CD3 and anti-4-1BB were added together, the concentration of RG7 was doubled. In Fig. 5 A and Fig. 5 B, the results of a typical experiment are shown for CD4<sup>+</sup> and CD8<sup>+</sup> T cells, respectively. The most notable feature



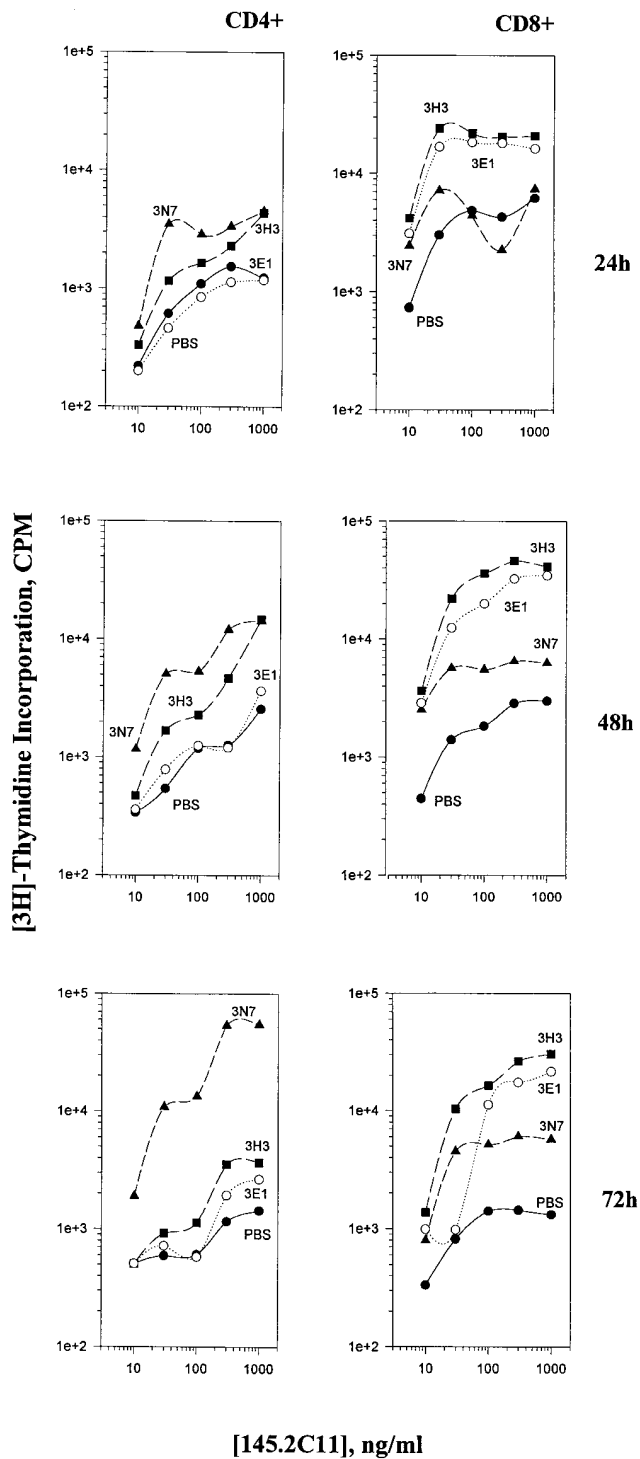
**Figure 3.** (A) Anti-4-1BB mAbs differentially costimulate in vitro proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets. Purified resting CD4<sup>+</sup> or CD8<sup>+</sup> BALB/c splenic T cells were cultured for 72 h in 96-well microtiter plates at  $1 \times 10^5$ /well with 0.5% APC  $\pm$  anti-4-1BB mAb at 10  $\mu$ g/ml and the indicated concentration of anti-CD3 mAb. Cells were pulsed with 0.5  $\mu$ Ci/well [<sup>3</sup>H]thymidine for the final 12 h of culture, harvested, and counted by liquid scintillation spectroscopy. (B) 4-1BB-Ig fusion protein inhibits anti-CD3-induced T cell proliferation. CD4<sup>+</sup> and CD8<sup>+</sup> T cells with APC were stimulated with anti-CD3 mAb at 300 ng/ml in the absence or presence of soluble 4-1BB-Ig fusion protein. Cells were pulsed during the final 12 h of culture with [<sup>3</sup>H]thymidine, harvested, and counted by liquid scintillation spectroscopy.

of this experiment is that previously activated CD8<sup>+</sup> T cells are more responsive to anti-CD3 stimulation when compared with CD4<sup>+</sup> T cells. This is not due to differential expression levels of CD3 within the two populations (our personal observations). Apart from the fact that CD8<sup>+</sup> T cells were more activated than CD4<sup>+</sup> T cells as judged by both the number and intensity of phosphoprotein bands on the gel, it is noteworthy that within the CD8<sup>+</sup> subset, signals delivered by anti-CD3 and anti-4-1BB are virtually indistinguishable from each other in terms of phosphotyrosine species observed, intensity of phosphorylation, as well as the kinetics of phosphorylation. In contrast, this is less true of the CD4<sup>+</sup> T cell population. In both figures, arrows delineate proteins undergoing significant changes in phosphorylation. The band with the slowest mobility also appears to undergo serine/threonine phosphorylation since its mobility is further retarded after 4-1BB cross-linking. Costimulation of CD8<sup>+</sup> T cells through CD3 and 4-1BB receptors alters the magnitude, kinetics, and profile of phosphoproteins compared with stimulation with either one alone. It should be pointed out that the data in both figures were generated from equivalent cell numbers from the same mice and that the number of cell equivalents (lysates) used/lane were identical in both experiments, as were the exposure times used in generating the ECL Western blots.

**Anti-4-1BB mAbs Enhance CTL Generation In Vivo.** Because anti-4-1BB mAbs had such a profound effect upon the signaling and proliferative responses of CD8<sup>+</sup> T cells, we questioned the importance of 4-1BB signaling in the generation of T cell effector functions such as cytotoxic T cell responses. Therefore, we measured the ability of a panel of anti-4-1BB mAbs of the IgG<sub>2A</sub> isotype in-

cluding blocking (3E1, 22B6, and 3H3) and nonblocking (1D8) mAbs to affect either the generation of CTL during acute GVHD or in a murine cardiac allograft or MHC mismatched skin transplant model. To generate acute GVHD and assay CTL activity, splenic T cells were isolated from BDF<sub>1</sub> (H-2<sup>dh</sup>) mice 10 d after intravenous injection of  $1-5 \times 10^7$  C57BL/6 (H-2<sup>b</sup>) splenocytes. Spleens removed from 1D8 and 22B6-treated GVHD mice were enlarged 3-5 times the size of normal spleens. Mice that received the isotype-matched negative control mAb 6E9 had spleens that were 2-3 times the size of normal mice. When assayed for CTL activity, 1D8 and 22B6, each known to bind to a different region of the 4-1BB molecule, enhanced CTL activity by nearly fourfold over that observed in control GVHD animals (Fig. 6 A). The powerful effect of anti-4-1BB mAbs on the enhanced development of CTL activity is further demonstrated in Fig. 6 B, which shows a marked reduction in the number of total viable splenocytes retrieved from mice treated with these antibodies. In addition, phenotypic analysis of splenocytes revealed that the percentage of CD8<sup>+</sup> T cells increased to 30% of the total cell number in mice treated with anti-4-1BB mAbs (Fig. 6 C), whereas GVHD mice injected with 6E9, the isotype-matched non-binding control or those receiving no antibody had 5-8% CD8<sup>+</sup> T cells. Epitope mapping studies of anti-4-1BB mAbs using 4-1BB fusion proteins in which domain swapping was carried out demonstrated that the 1D8 mAb was unique in that it bound to the membrane proximal region of the extracellular domain of the 4-1BB molecule not involved in 4-1BBL binding.

**Anti-4-1BB mAbs Enhance Cardiac Allograft Rejection.** 3E1 was chosen to study for its ability to affect cardiac allograft



**Figure 4.** Anti-CD28 (3N7) and anti-4-1BB (3H3 and 3E1) mAbs costimulate opposite T cell subsets. Purified CD4<sup>+</sup> and CD8<sup>+</sup> resting T cells were costimulated with either anti-CD3 and anti-CD28 or anti-CD3 and anti-4-1BB mAbs for 24, 48, or 72 h. Cultures were pulsed with [<sup>3</sup>H]thymidine for the final 12 h of culture.

rejection in mice because its avidity was the highest of all of the anti-4-1BB mAbs that we generated and it most efficiently blocked 4-1BBL binding to its receptor. The results of this experiment clearly demonstrated that the binding of

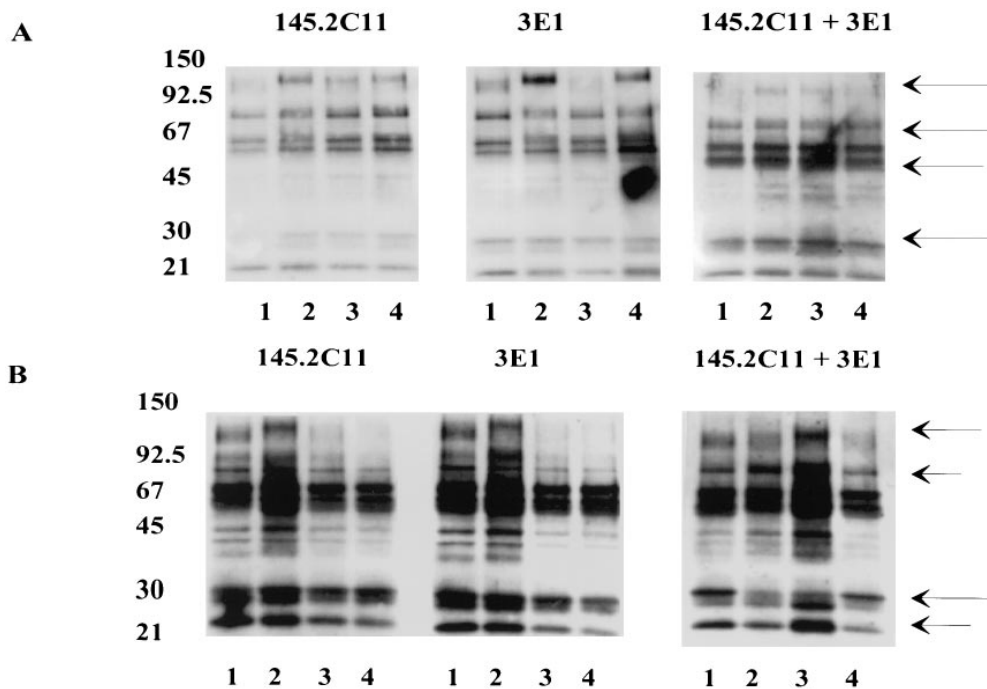
anti-4-1BB mAb to activated T cells in mice receiving heart allografts led to the rapid rejection of the allograft (3 of 5 by day 7–8) compared with the control group (3 of 4 by day 10). Similar results were obtained in MHC-mismatched skin transplant experiments in mice using 3E1, 3H3, or 1D8 (data not shown).

**Anti-4-1BB Costimulation Induces IFN- $\gamma$  Production in CD8<sup>+</sup> T Cells.** To understand better how 4-1BB-mediated signals amplify CTL activity, we analyzed the kinetics of IL-2, IL-4, IL-10, and IFN- $\gamma$  production by resting CD4<sup>+</sup> and CD8<sup>+</sup> or unseparated T cells stimulated with anti-CD3 or anti-CD3 together with anti-CD28 or anti-4-1BB. In Fig. 7, it can be seen that CD28-mediated costimulation significantly amplified the production of all four lymphokines by CD4<sup>+</sup> and unseparated T cells but had little effect on CD8<sup>+</sup> T cells. In contrast, 4-1BB-mediated costimulation had no significant effect upon the CD4<sup>+</sup> T cell subset with perhaps IFN- $\gamma$  being the exception. On the other hand, 4-1BB-mediated costimulation markedly enhanced the production of IFN- $\gamma$  by CD8<sup>+</sup> T cells as well as in unseparated T cells. This feature appeared to be a general characteristic of anti-4-1BB mAbs, as several of them were tested and found to have the same properties (data not shown).

## Discussion

In this study, we have confirmed that anti-4-1BB mAb-induced cross-linking of the 4-1BB receptor *in vitro* provides a potent costimulatory effect on anti-CD3-induced polyclonal T cell proliferation. However, we also found that 4-1BB-mediated proliferation was highly biased toward the CD8<sup>+</sup> subset of T cells. For instance, the addition of anti-4-1BB mAb 3H3 (10  $\mu$ g/ml) reduced the threshold of anti-CD3 from 1,000 ng/ml to 10 ng/ml and increased the absolute value of cpm of [<sup>3</sup>H]thymidine incorporated (Fig. 2 B) by CD8<sup>+</sup> T cells 100-fold. In contrast, signaling through the 4-1BB receptor on CD4<sup>+</sup> T cells resulted in a fourfold increase in proliferative capacity (Fig. 2 A). In both cases, 4-1BB-mediated costimulation was maximal at 48 h. Stimulation of either population with varying doses of anti-CD3 alone demonstrated that these two populations are near-equivalent in their ability to respond to CD3-mediated proliferative signals, with CD4<sup>+</sup> T cells being somewhat more responsive in these experiments. Furthermore, in contrast with 4-1BB, CD28-mediated costimulation was highly biased toward CD4<sup>+</sup> T cell activation (Fig. 5) and optimal activation occurred at 72 h. Thus, there is a reciprocal relationship of costimulation through these two receptor systems with regard to T cell subset proliferation.

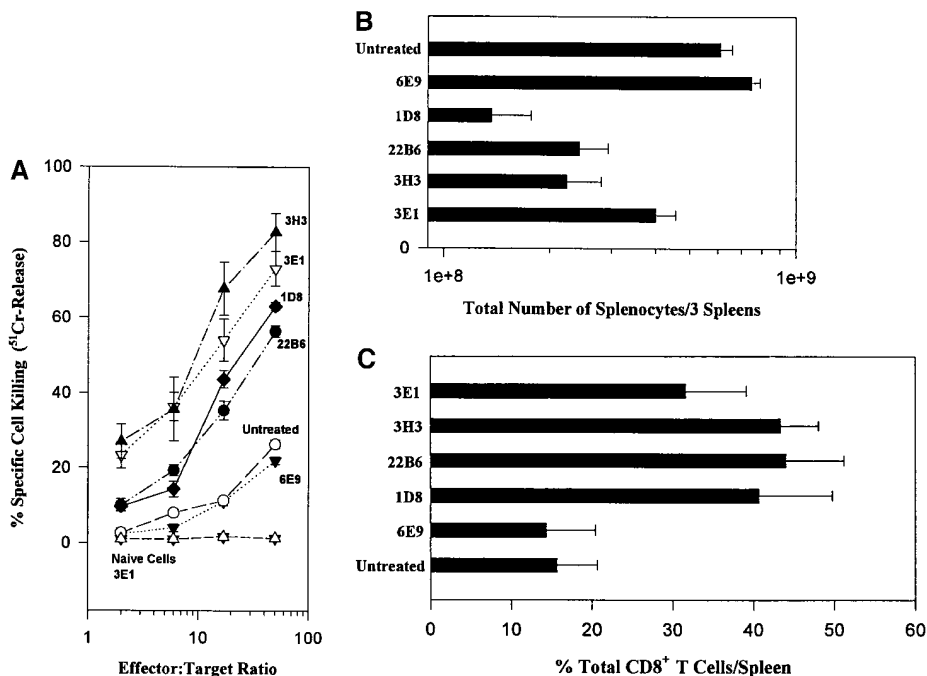
The ability of anti-4-1BB mAbs to enhance T cell activation was found to be more dependent upon epitope recognition than antibody isotype or antibody avidity, because both antibodies of the IgM and IgG<sub>2A</sub> isotype were equally effective as costimulators, and within the IgG<sub>2A</sub> isotype, antibodies having similar avidity varied markedly with respect to their biological activity. For instance, all of the antibodies, regardless of avidity or isotype, that bound near or at



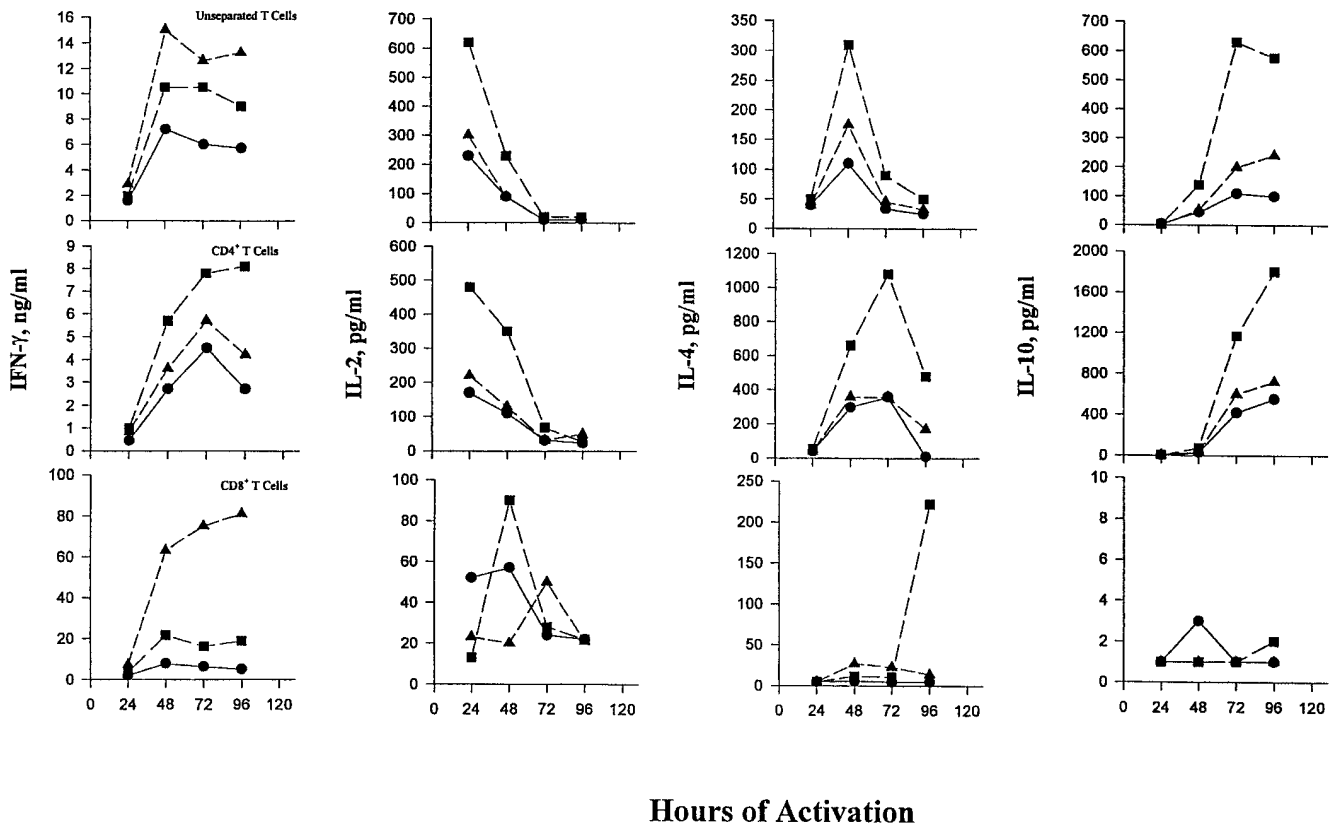
**Figure 5.** Cross-linking 4-1BB initiates distinct patterns and kinetics of protein tyrosine phosphorylation in CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets. BALB/c splenocytes were activated as described earlier. CD4<sup>+</sup> (A) and CD8<sup>+</sup> (B) subsets were incubated with 10 ng/ml of anti-CD3, anti-4-1BB (3E1), or both at 10 ng/ml each. After 15-min incubation at room temperature, the cells were equilibrated to 37°C and cross-linked with 50 ng/ml of RG7, a mouse anti-rat IgG that cross-reacts with hamster IgG. Where both anti-CD3 and anti-4-1BB were added together, RG7 was added at 100 ng/ml. Untreated (lane 1), 1 min (lane 2), 3 min (lane 3), 9 min (lane 4).

the ligand binding site were most effective at inducing co-stimulation. This observation led us to question whether or not enhanced proliferation induced by these reagents was a consequence of their ability to block a negative proliferation signal transmitted by 4-1BBL binding to the 4-1BB receptor. This was not found to be the case (Fig. 3 B). Rather, our data support the view that cross-linking the 4-1BB receptor on an activated T cell transmits a positive

activation signal to the cell. This notion is further supported by the observation that 4-1BB cross-linking on activated CD8<sup>+</sup> T cells led to rapid tyrosine phosphorylation of multiple substrates and that the pattern of phosphorylation was nearly identical to anti-CD3-induced protein tyrosine phosphorylation. This finding suggests that the 4-1BB receptor on CD8<sup>+</sup> T cells may be structurally or functionally coupled to the TCR of these cells.



**Figure 6.** (A) Anti-4-1BB mAbs enhance or inhibit in vivo generation of CTL responses during GVHD responses. GVHD was established by intravenous injection of  $5 \times 10^7$  C57BL/6 (H-2<sup>b</sup>) splenocytes into DBA/2 (H-2<sup>d</sup>) mice as described in the Materials and Methods. The administration of mAbs was carried out as described earlier. On day 10, spleens were collected from GVHD mice and single cell suspensions prepared. CTL activity in this population was measured directly by assaying CTL-mediated killing of <sup>51</sup>Cr-labeled P815 cells. (B) Total splenocyte levels are markedly reduced in mice receiving anti-4-1BB mAbs. The total number of viable splenocytes obtained from groups of three spleens pooled from each treatment group was assessed by microscopy and trypan blue exclusion. (C) Percentages of viable CD8<sup>+</sup> T cells were determined for each treatment group using FITC-conjugated anti-murine CD8 mAb (PharMingen) and flow cytometry.



**Figure 7.** Costimulation of CD8<sup>+</sup> T cells with anti-4-1BB but not anti-CD28 leads to IFN- $\gamma$  production. Unseparated and CD4<sup>+</sup> or CD8<sup>+</sup> resting T cells were stimulated with anti-CD3 (●) or anti-CD3 and either anti-CD28 (■) or the anti-4-1BB mAb 3H3 (▲). Activated T cell culture SN were collected at 24, 48, 72, and 96 h and assayed by ELISA for IL-2, IL-4, IL-10, and IFN- $\gamma$ .

In light of the fact that CD8<sup>+</sup> T cells preferentially respond to 4-1BB costimulation, we assessed the effects of several anti-4-1BB mAbs *in vivo*. For these studies, we chose to examine the effects of anti-4-1BB mAbs on CTL development as this activity represents a major function of the CD8<sup>+</sup> T cell subset. To facilitate these studies, we chose acute GVHD, cardiac allograft, and MHC-mismatched skin transplantation as murine models for our studies. The ability of anti-4-1BB mAbs to enhance GVHD (Fig. 6 A) leading to marked depletion of host splenocytes (Fig. 6 B) appears to be the result of preferential costimulation and amplification of CD8<sup>+</sup> T cells *in vivo* (Fig. 6 C). In contrast, the percentage of CD4<sup>+</sup> T cells did not change (data not shown). In cardiac allograft (Fig. 7) and skin transplant studies (data not shown) we found that transplants were rejected faster in mice treated with any of a variety of anti-4-1BB mAbs than what was observed in control mice injected with the 6E9 mAb. We also found certain functional differences in this system. For example, certain mAbs, such as 22B6 and 1D8, were poor inducers of proliferation, whereas mAbs such as 3E1 and 3H3 were strong inducers of proliferation. Nevertheless, all were equally effective at enhancing GVHD in mice and in rejecting MHC-mismatched skin transplants (data not shown). This suggests that the induction of CD8<sup>+</sup> T cell proliferation was not critical to the observed func-

tional effects in these systems. To explore further this issue, we examined lymphokine production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells or unseparated T cells costimulated with anti-CD3 alone or via costimulation with either anti-4-1BB or anti-CD28 over a 96-h time course. The results of this experiment demonstrated that anti-4-1BB costimulation had a profound effect upon the upregulation of IFN- $\gamma$  production by CD8<sup>+</sup> T cells but had little or no effect upon IL-2, IL-4, or IL-10 production in either subset. In contrast, anti-CD28-mediated costimulation had only a slight effect upon IFN- $\gamma$  production by either subset. In contrast, anti-CD28 costimulation markedly enhanced IL-2, IL-4, and IL-10 production in CD4<sup>+</sup> T cells. When unseparated T cells were activated as described above, the only notable difference was the level of IFN- $\gamma$  produced after anti-4-1BB costimulation. Allowing that ~30–35% of these cells were CD8<sup>+</sup>, on a cell by cell basis, CD8<sup>+</sup> T cells in these cultures produced lower (~50%) levels of IFN- $\gamma$ , which was probably caused by inhibition of Th<sub>1</sub> cytokine production by IL-4 and IL-10.

From these studies, it would appear that 4-1BB/4-1BBL differs from other previously defined receptor–ligand systems known to be important for costimulation of T cells in that its primary effect appears to be the regulation of CD8<sup>+</sup> T cell activation. In contrast, other systems such as CD28–



CD80/CD86, CD27/CD27L, or gp39/CD40 primarily affect the function of CD4<sup>+</sup> T cells. Finally, the increase in amplitude of the CTL response during GVHD, cardiac allograft, and skin rejection is not just limited to these forms of alloantigenic stimulation; we have also observed the same type of phenomena in mice injected with either non-metastatic or highly metastatic, poorly immunogenic tu-

mors (manuscript in preparation). Under these conditions, the administration of anti-4-1BB promoted rejection of large established tumors and markedly enhanced animal survival. Taken together, these observations point to a potential therapeutic role for this receptor in the management of cancer and chronic infectious diseases.

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