The Journal of Immunology

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J Immunol 2001; 167:1313-1324; ;
doi: 10.4049/jimmunol.167.3.1313
http://www.jimmunol.org/content/167/3/1313

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

4-1BB Ligand Induces Cell Division, Sustains Survival, and Enhances Effector Function of CD4 and CD8 T Cells with Similar Efficacy[1]

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A costimulatory member of the TNFR family, 4-1BB, is expressed on activated T cells. Although some reports have suggested that 4-1BB is primarily involved in CD8 T cell activation, in this report we demonstrate that both CD4 and CD8 T cells respond to 4-1BB ligand (4-1BBL) with similar efficacy. CD4 and CD8 TCR transgenic T cells up-regulate 4-1BB, OX40, and CD27 and respond to 4-1BBL-mediated costimulation during a primary response to peptide Ag. 4-1BBL enhanced proliferation, cytokine production, and CTL effector function of TCR transgenic T cells. To compare CD4 vs CD8 responses to 4-1BBL under similar conditions of antigenic stimulation, we performed MLRs with purified CD4 or CD8 responders from $CD28^{+/+}$ and $CD28^{-/-}$ mice. We found that CD8 T cells produced IL-2 and IFN-γ in a 4-1BBL-dependent manner, whereas under the same conditions the CD4 T cells produced IL-2 and IL-4. 4-1BBL promoted survival of CD4 and CD8 T cells, particularly at late stages of the MLR. CD4 and CD8 T cells both responded to anti-CD3 plus s4-1BBL with a similar cytokine profile as observed in the MLR. CD4 and CD8 T cells exhibited enhanced proliferation and earlier cell division when stimulated with anti-CD3 plus anti-CD28 compared with anti-CD3 plus 4-1BBL, and both subsets responded comparably to anti-CD3 plus 4-1BBL. These data support the idea that CD28 plays a primary role in initial T cell expansion, whereas 4-1BB/4-1BBL sustains both CD4 and CD8 T cell responses, as well as enhances cell division and T cell effector function. ***The Journal of Immunology,*** **2001, 167: 1313–1324.**

The interaction of CD28 on T cells with its ligands B7-1 and B7-2, together with signals through the TCR provides important signals for initial T cell activation (1, 2). Subsequently to these initial events, a number of additional receptor-ligand pairs are up-regulated on the T cell and APCs, respectively. These receptor/ligands may be involved in sustaining, diversifying, and/or amplifying the immune response. In particular, members of the TNFR/TNF ligand family, including 4-1BB/4-1BB ligand (4-1BBL),[4] CD27/CD70, and OX40/OX40 ligand (OX40L) appear to be important in enhancing T cell responses subsequent to initial activation (3–6). 4-1BB is a member of the TNFR family expressed on activated CD4 and CD8 T cells (3). 4-1BBL is expressed on activated APC including B cells, macrophages, and dendritic cells (7–9).

4-1BBL has been shown to costimulate T cell responses independently of signals through the CD28 molecule (9–11) and can stimulate both primary (12, 13) and secondary (12, 14–16) responses of both CD4 (10, 13, 14, 16) and CD8 T cells (12, 17–20). Ligation of 4-1BB by anti-4-1BB Abs or 4-1BBL promotes the development of CTL activity and anti-tumor immunity (12, 17, 18, 21, 22). In vitro, 4-1BB-mediated costimulation has been shown to sustain Th1 T cell responses and prevent activation-induced death (15, 16). $4\text{-}1BBL^{-/-}$ mice revealed that 4-1BB/4-1BBL interaction plays a role in the CTL response to influenza virus as well as in skin allograft rejection. 4-1BBL is not required for the neutralizing Ab response to vesicular stomatitis virus or to clear lymphocytic choriomeningitis virus (LCMV) (12, 19). However, the LCMV-specific response has been shown to become 4-1BBL-dependent under conditions of suboptimal antigenic stimulation (12, 20).

Studies using a particular collection of anti-4-1BB Abs have suggested that 4-1BB is preferentially involved in the expansion of CD8 T cells and that it has a much smaller effect on CD4 T cells (21, 23). In contrast, a number of studies have shown a substantial effect of 4-1BB/4-1BBL on CD4 T cells (10, 13, 14, 16, 24). For example, DeBenedette et al. (14) used an MHC class II restricted autoreactive T cell hybridoma as well as purified CD4 T cells to demonstrate that 4-1BBL could provide a costimulatory signal for T cell activation on APC lacking B7 molecules. Follow-up experiments by Chu et al. (10) used purified CD4 T cells responding to allogeneic stimulator cells expressing 4-1BBL to demonstrate that 4-1BBL could augment cytokine production by T cells independently of signals through the CD28 molecule. These studies were extended by Gramaglia et al. (13), who used MHC class II-restricted, pigeon cytochrome *c* (PCC)-specific TCR transgenic T cells to show that 4-1BBL can augment cytokine production and T cell proliferation in both the primary and secondary responses to Ag. Furthermore, Cannons et al. (24) have used purified CD4 T cells responding to immobilized anti-CD3 plus immobilized 4-1BBL to show that 4-1BBL-dependent cytokine production is

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Received for publication February 12, 2001. Accepted for publication May 29, 2001.

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[1] This work was funded by a grant from the Canadian Institutes for Health Research (to T.H.W.). J.L.C. was funded by a Canadian Institutes for Health Research doctoral award.

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[4] Abbreviations used in this paper: 4-1BBL, 4-1BB ligand; LCMV, lymphocytic choriomeningitis virus; AP, alkaline phosphatase; PCC; pigeon cytochrome *c*; s4-1BBL, soluble 4-1BBL; OX40L, OX40 ligand; GVHD, graft-versus-host disease; TRAF, TNFR-associated factor.

0022-1767/01/$02.00

dependent on the p38 mitogen-activated protein kinase. Thus, there are extensive data arguing that 4-1BBL can stimulate CD4 T cells. The present studies were undertaken to compare CD4 vs CD8 T cell expression of 4-1BB and response to 4-1BBL under comparable conditions of antigenic stimulation.

Materials and Methods

Mice, cell lines, Abs, and reagents

C57BL/6 and BALB/c mice were obtained from Charles River Breeding Laboratories (St. Constant, Quebec, Canada) and used at 8–12 wk of age. $CD28^{-/-}$ mice backcrossed on the H-2^b background ($n = 10$) (25) were obtained from Dr. T. Mak (Ontario Cancer Institute, Toronto, Ontario, Canada). The $CD28^{-/-}$ mice on the BALB/c background were obtained from The Jackson Laboratory (Bar Harbor, ME). Genotyping for CD28 mutation was performed by PCR as described (12). P14 transgenic mice, expressing a TCR specific for the LCMV gp33–41 presented in association with H-2^b on both a C57BL/6 $CD28^{+/+}$ (26) and $CD28^{-/-}$ background (27), were obtained from Dr. P. Ohashi (Ontario Cancer Institute, Toronto, Canada) and bred in the University of Toronto animal facility. DO11.10 TCR transgenic mice (H-2^d) (28) were obtained from Dr. R. Miller (Ontario Cancer Institute) and were crossed with BALB/c $CD28^{-/-}$ mice in our facility. All animal protocols were approved by the University of Toronto Animal Care Committee according to the guidelines of the Canadian Council on Animal Care. The LCMV glycoprotein-related weak agonist peptide A4Y (KAVANFATM) was synthesized at the Amgen Institute (Boulder, CO) as described (27). The OVA peptide (323–339) (ISQAVHAAHAEINEAGR) (29) was synthesized by Dr. J. Gariépy (Ontario Cancer Institute).

The EL4:4-1BBL transfectant was described by DeBenedette et al. (12). The BALB/c B cell lymphomas M12 4.1 and K46J were originally described by Kim et al. (30). K46J lymphomas constitutively express high levels of 4-1BBL, undetectable CD80, and low levels of CD86 (14). A variant of the BALB/c B lymphoma M12 4.1, when treated overnight with cAMP to up-regulate costimulatory molecules, was found to express low levels of 4-1BBL but moderate levels of CD80 and high levels of CD86 (24).

The anti-CD3 producing hybridoma 145-2C11 was provided by Dr. J. Bluestone (University of Chicago, Chicago, IL). The hybridomas N418 (anti-CD11c), Y-3P (anti-A^b), MKD6 (anti-A^d), RA3-6B2 (anti-B220), TIB-128 (anti-MAC-1), M1/69 (anti-heat-stable Ag), RG7/7.6H2 (anti-rat Ig κ-chain), and the IL-2-dependent line CTLL were obtained from the American Type Culture Collection (Manassas, VA). The anti-CD28-secreting hybridoma 37.51.1 (31) was provided by Dr. J. Allison (University of California, Berkeley, CA). Anti-4-1BB Ab, 3H3 (22), was provided by Dr. R. Mittler, (Emory University, Atlanta, GA). A cell line producing CTLA4.Ig was provided by Dr. P. Lane (University of Birmingham, Birmingham, U.K.). The anti-DO-11.10 TCR-secreting hybridoma (KJ1.26.1) was obtained from Dr. P. Marrack (National Jewish Hospital, Denver, CO) (32) and was used to monitor transgene expression of the DO-11.10 mice. Cells were maintained in RPMI 1640 containing 10% FCS (Cansera, Rexdale, Ontario, Canada), 50 μM 2-ME, MEM nonessential amino acids (Life Technologies, Gaithersburg, MD), antibiotics, pyruvate, and glutamine as previously described (33).

CTLA4.Ig and the Abs described above were purified from hybridoma supernatants using protein G- or protein A-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. 3T3 cells secreting 4-1BB linked to alkaline phosphatase (AP) were provided by Dr. B. Kwon (Indiana University, Indianapolis, IN) (8). 4-1BB.AP was purified on anti-AP-Sepharose as previously described (8). AP from human placenta was obtained from Sigma (St. Louis, MO). A recombinant baculovirus encoding a soluble form of 4-1BBL was used to produce soluble 4-1BBL (s4-1BBL) in insect cell supernatants as previously described (11). s4-1BBL was purified from the insect cell supernatants by affinity chromatography using the 4-1BBL-specific mAb, TKS-1 (34). s4-1BBL was eluted from the affinity column in 0.1 M Na_2HPO_4, 0.15 M NaCl, pH 11.3 and immediately neutralized using 20% glacial acetic acid. Protein concentration was determined using the method of Lowry.

Lymphocyte isolation

For T cell isolation, APCs were depleted from cell suspensions from spleen and lymph node in HBSS (Life Technologies)/2.5% FCS/50 μM 2-ME, with a mixture of Abs including anti-MHC class II, anti-B220, anti-heat-stable Ag, anti-MAC-1, and anti-CD11c each at a final concentration of 10 μg/ml at 4°C for 30 min. A 1/10 dilution of baby rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) was added, and the cultures were incubated at 37°C for 40 min. To remove adherent cells, the cell suspensions were passed through a Sephadex G10/nylon wool column and then centrifuged through Percoll gradients consisting of 60, 70, and 80% Percoll layers. Small (high density) resting T cells were isolated from the 70/80% interface and used for subsequent experiments.

MLRs

CD4 and CD8 T cells were isolated from the spleen and lymph nodes of $CD28^+$ and $CD28^-$ mice by negative selecting columns from Cytovax Biotechnologies (Edmonton, Alberta, Canada). The purity of the populations was assessed by flow cytometry and was found to be at least 90%. The M12 4.1 cells were treated overnight with dibutyryl-cAMP at a final concentration of 300 μM to induce B7 family molecules as previously described (35). Stimulator B lymphomas (M12 4.1 or K46J) were irradiated (10,000 rad) to prevent their proliferation. Primary MLR cultures were performed in 24-well plates consisting of 1×10^6 T cells and 5×10^5 B lymphomas in a total volume of 1.5 ml. After 3 days, cultures were fed by replacing 250 μl of supernatant with fresh medium. After a total of 3, 5, or 7 days of incubation, 1 ml of culture supernatant was removed and frozen immediately at −70°C. For the inhibition of cytokine production, MLR cultures were stimulated as described above but with the addition of soluble reagents.

Cytokine assays

IL-2 was detected using the indicator cell line CTLL as described (10, 24). Serial dilutions of the culture supernatant were prepared in triplicate and incubated with 1×10^4 indicator cells in 100 μl in 96-well plates for 24 h. During the final 8 h, the cells were labeled with [^{3}H]thymidine (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). Cultures were harvested and analyzed on the TopCount 96-well liquid scintillation counter (Canberra-Packard, Meriden, CT). ELISA was performed on diluted supernatants from the cultures, using pairs of anti-murine IFN-γ mAbs or anti-murine IL-4 mAbs purchased from PharMingen (San Diego, CA) according to the manufacturer's instructions.

Primary responses of TCR transgenic LCMV-specific T cells in vitro

T cells were isolated from either $CD28^+$ or $CD28^-$ P14 transgenic mice, expressing a transgenic TCR specific for LCMV gp as previously described (12). Purified resting T cells were cultured with either irradiated (10,000 rad) EL4 or EL4:4-1BBL cells, plus 0.2 μM of the LCMV gp related A4Y peptide. Following 3 days of in vitro stimulation, CTL activity was assayed against A4Y peptide-pulsed EL4 or EL4:4-1BBL targets in a conventional ^{51}Cr release assay. After 5 h, 70 μl of supernatant was harvested and measured in a TopCount scintillation counter. Maximum and spontaneous release was determined from wells that contained targets in the presence of either 1% SDS or medium alone, respectively. Specific lysis was calculated as [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)] × 100. Spontaneous release was routinely 5–6% of maximum ^{51}Cr release for each assay.

Immunofluorescent staining

Staining was performed in PBS with 2% FCS and 0.2% NaN_3. FITC-labeled Abs were labeled in our laboratory, whereas PE and APC-labeled reagents were purchased as indicated below. The following Abs and fluorescent reagents were used: CD4-FITC, CD8-FITC, CD3-FITC, B220-FITC, Mac-1-FITC, OX40-PE (Serotec, Raleigh, NC), 4-1BB-PE (PharMingen), and CD27-PE (PharMingen). All isotype control Abs were purchased from PharMingen. Propidium iodine staining and forward and side scatter were used to gate on live cells. Flow cytometry was conducted using a FACSCalibur (BD Biosciences, Mountain View, CA), and data were analyzed with CellQuest software (BD Biosciences, San Jose, CA).

Labeling of cells with fluorescent dyes

T cells were stained with CFSE (Molecular Probes, Eugene, OR) as previously described (36). In brief, cells were resuspended in PBS at 5×10^7/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 1640 with 20% FCS and recounted.

Results

Expression of 4-1BB, OX40, and CD27 during a primary peptide-specific T cell response of CD4 and CD8 T cells

Previous studies have demonstrated that following anti-CD3 stimulation or anti-CD3 plus cytokine stimulation, 4-1BB can be induced on both CD4 and CD8 T cells (8, 37). Following anti-CD3 treatment of total splenocytes, 4-1BB is readily detectable by 24 h with maximal surface expression between 48 and 72 h, declining by 110 h (3). To assess the kinetics of 4-1BB expression during a primary response to specific Ag, we used two different TCR transgenic models: the D^b/LCMV gp-specific P14 transgenic mouse (26) to evaluate a CD8 T cell response and the A^d/$OVA_{323–339}$-specific DO-11.10 transgenic mouse (28) to evaluate a CD4 T cell response. Total splenocytes from the TCR transgenic mice were stimulated with concentrations of peptide that induce maximal T cell proliferation. 4-1BB expression was assessed by flow cytometry after 24, 48, 72, and 96 h of culture. In parallel, T cells were also assessed for CD27 and OX40 expression. CD27 is a member of the TNFR family expressed constitutively on T cells, whereas OX40, like 4-1BB, is expressed only on activated T cells (5). For the CD4 TCR transgenic T cells, 4-1BB expression was detected between 24 and 48 h with sustained or increased expression by 96 h. Use of a 10-fold higher concentration of peptide resulted in indistinguishable kinetics of induction of all three TNFR family members (data not shown). Analysis of the CD8 TCR transgenic T cells revealed a more transient expression pattern for 4-1BB (Fig. 1*A*), whereas CD27 levels were maintained throughout the time course of the experiment (Fig. 1*C*). Interestingly, OX40 staining

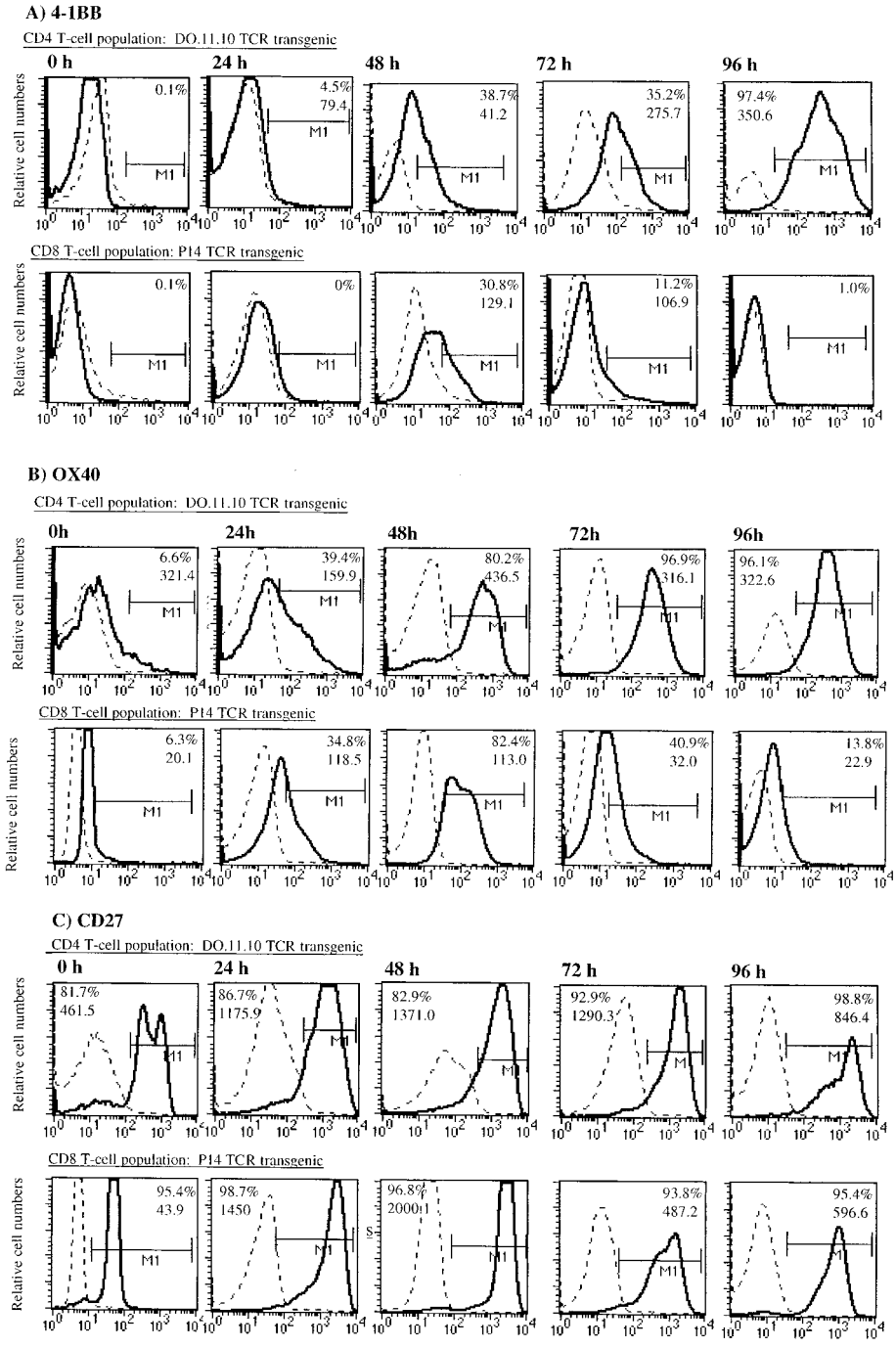

FIGURE 1. Expression of TNFR family members on CD4 and CD8 T cells. Total splenocytes from DO-11.10 TCR transgenic mice were stimulated with 2 μM $OVA_{323–339}$ Y peptide and analyzed after 0, 24, 48, 72, and 96 h of culture. Cells were gated on live cells based on PI and CD4 staining (*upper panel*). Total splenocytes from LCMV P14 TCR transgenic mice were stimulated with 0.5 μM p33 peptide and analyzed at 0, 24, 48, 72, and 96 h of culture. Cells were gated on live cells based on PI and CD8 staining (*lower panel*). Cells were stained for expression of 4-1BB (*A*), OX40 (*B*), and CD27 (*C*). The dotted line represents the isotype control and the bold line the specific staining in each panel. Numbers in each panel indicate percentage of cells staining and mean fluorescent intensity of the gated population. This experiment is representative of three individual experiments.

showed a similar trend: both CD4 and CD8 T cells can be induced to express OX40 during a primary response to their specific Ag, but the expression of OX40 was more transient on the MHC class I-specific T cells (Fig. 1*B*). Thus both CD4 and CD8 T cells appear to be able to express OX40 and 4-1BB after activation. Furthermore, the entire T cell population expressed OX40 and CD27 after activation, and a major fraction of the T cells also expressed 4-1BB. These results imply that individual T cells can express all three TNFR family members simultaneously.

4-1BBL augments both expansion and effector function of P14, LCMV-specific, TCR transgenic T cells during the primary response to Ag

The LCMV-specific T cell response has previously been shown to be relatively independent of CD28-mediated costimulation. However, when a weak peptide ligand is used, this response becomes more dependent upon CD28 (38). Our previous results demonstrated that $CD28^+$ T cells responding to a wild-type LCMV glycoprotein peptide (p33) are insensitive to costimulation with either 4-1BBL or anti-CD28. However, the CTL response became sensitive to 4-1BBL upon stimulation with the weaker ligand A4Y peptide, particularly in the absence of CD28 (12). In this report, we have further evaluated this model to determine whether this effect of 4-1BBL was at the level of CD8 T cell expansion, due to an increase in effector cell function, or both. Fig. 2*A* demonstrates that by 48 h of culture, the presence of 4-1BBL on the stimulating EL4 cells enhanced proliferation of $CD28^+$ and $CD28^-$ P14 TCR transgenic T cells. This enhancement was blocked upon the addition of a soluble form of 4-1BB (4-1BB.AP). This 4-1BBL-dependent T cell expansion was also accompanied by increased IFN-γ secretion by both the $CD28^+$ and the $CD28^-$ T cells (Fig. 2*B*). Addition of 4-1BB.AP, but not control AP, to the cultures reduced the level of IFN-γ produced to that observed when cells were stimulated with EL4 cells alone (Fig. 2*B*).

After 3 days of culture, the T cells were counted to normalize for T cell expansion during the first 3 days of culture, and then equal numbers of T cells were evaluated for their ability to kill EL4 target cells. Fig. 2*C* shows that $CD28^-$ LCMV gp-specific P14 T

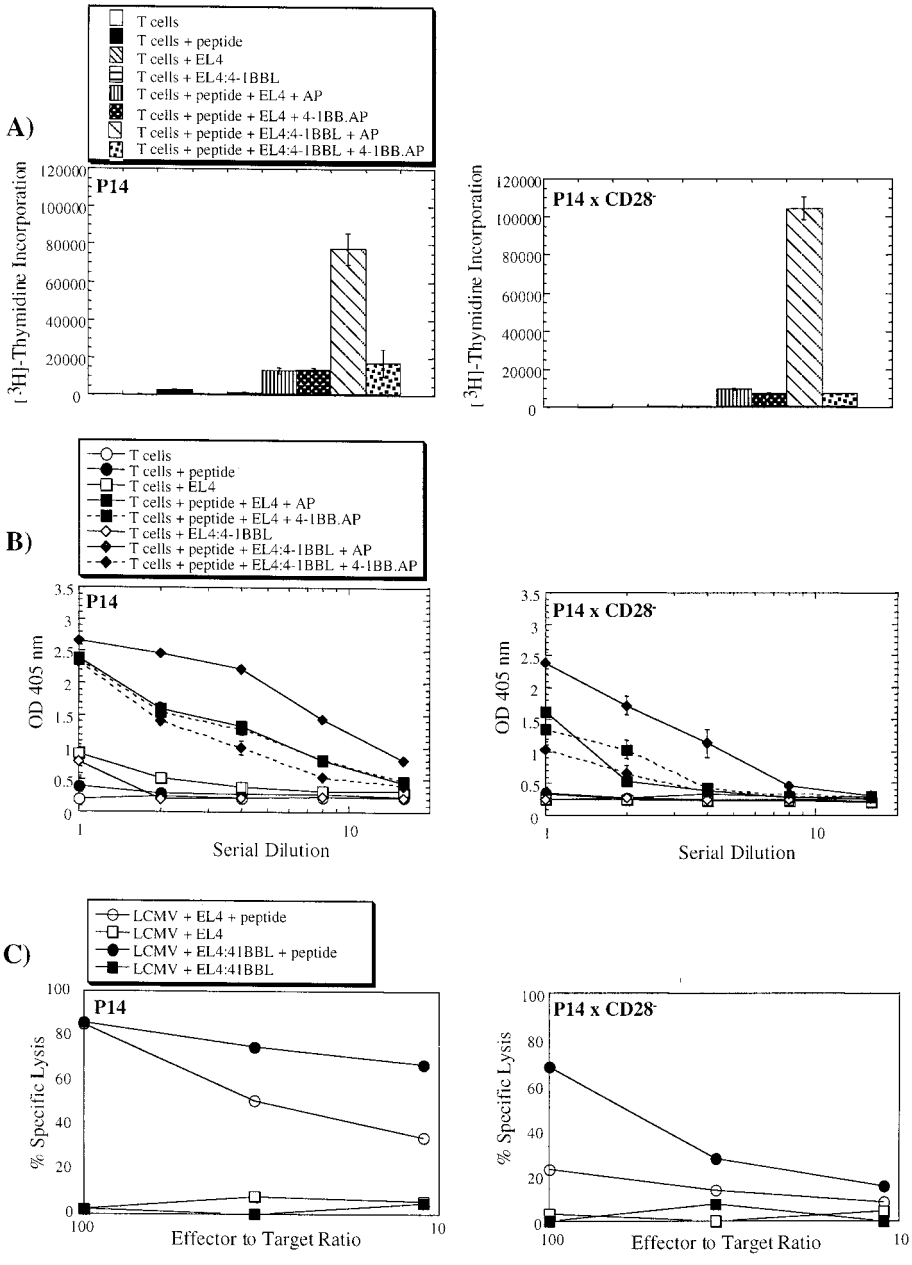

FIGURE 2. A role for 4-1BBL in CD8 T cell expansion and effector function. T cells were purified from P14 LCMV gp-specific and $CD28^{-/-}$ × P14 LCMVgp-specific TCR transgenic mice. T cells (1×10^6/ml) were cultured with 0.2 μM A4Y peptide and 1×10^5/ml irradiated EL4 or EL4:4-1BBL cells for 3 days. Where indicated, 10 μg/ml AP control or 4-1BB.AP (to block 4-1BBL-4-1BB interaction) were added at the onset of culture. *A*, T cell proliferation on day 2 of culture via [^{3}H]thymidine incorporation. *B*, Supernatant was removed from the culture on day 3 and evaluated for IFN-γ levels by ELISA. *C*, T cells were washed, counted (5×10^6/ml), and tested for killing of ^{51}Cr-labeled A4Y peptide-pulsed EL4 targets. Results presented are the average of triplicate wells and are representative of two individual experiments.

cells responded poorly to A4Y peptide presented by EL4 alone but exhibited an enhanced primary CTL response to A4Y peptide presented by 4-1BBL-transfected EL4 cells. Furthermore, this response was blocked by inclusion of soluble 4-1BB receptor (data not shown). Although 4-1BB.AP blocked the development of the CTL effectors, inclusion of 4-1BB.AP during the 5-h ^{51}Cr release assay had no effect on killing. This indicates that 4-1BBL acted at the level of development of the CTL effectors rather than in the actual killing of targets (Ref. 12 and data not shown). In Fig. 2, *B* and *C*, the CD28$^+$ P14 transgenic T cells showed a slightly higher response to EL4:4-1BBL than did the CD28$^-$ T cells. This is attributed to the observation that the transfected EL4 cells also express some B7.1 (12). Taken together, the results in Fig. 2 demonstrate that 4-1BBL induced a modest increase in T cell proliferation as well as enhancing the effector function per T cell during the primary response to Ag.

4-1BBL augments the primary response of DO-11.10 TCR transgenic T cells

Given that 4-1BB is expressed during primary stimulation of the MHC II-restricted DO-11.10 T cells, it was also of interest to determine the effect of 4-1BBL on the DO-11.10 T cell response. Purified DO-11.10 T cells from CD28$^{+/+}$ or CD28$^{-/-}$ TCR transgenic mice were stimulated with a B cell lymphoma, K46J, previously shown to express high levels of 4-1BBL and little or no B7 family molecules (14). We observed that the response of DO-11.10 TCR transgenic T cells was relatively independent of costimulation at high doses of peptide (data not shown). Therefore, to test the 4-1BBL sensitivity of the DO-11.10 response, we used a suboptimal concentration of OVA$_{323–339}$ peptide that is in the midpoint of the dose-response curve for this T cell. Fig. 3*A* demonstrates that CD28$^+$ DO-11.10 T cells produced IL-2 in response to OVA$_{323–339}$ presented by K46J lymphoma, and IL-2 production was blocked by 4-1BB.AP but not by AP control. Similarly, CD28$^-$ T cells also produced IL-2 in a 4-1BB-dependent manner, although the level of IL-2 produced was lower. Fig. 3*B* shows that by 72 h, CD28$^+$ but not CD28$^-$ DO-11.10 T cells produced a minimal amount of IFN-γ, and this response was also inhibited by soluble 4-1BB. IL-4 was not detected in the culture supernatants at 48 or 72 h by ELISA (data not shown). These results demonstrate that 4-1BBL can costimulate CD4 T cells in a CD28-independent manner during a primary Ag-specific response.

Comparison of CD4 vs CD8 T cell responses to 4-1BBL and CD28-mediated costimulation during an MLR

The results with TCR transgenic T cells suggest that both CD4 and CD8 T cells can respond to 4-1BBL-mediated costimulation even in the absence of a CD28 signal. However, it is difficult to compare the relative importance of 4-1BB/4-1BBL for the CD4 vs CD8 T cells using cells from TCR transgenic mice, given that the affinity of peptide for MHC and of MHC/peptide for TCR may well influence the outcome of the results. In an allogeneic T cell response in which MHC molecules are mismatched across the MHC, a high proportion of T cells respond to MHC/peptide differences and the response likely involves a large number of different receptors. Therefore, it seems reasonable to assume that the response will be similar in magnitude for the MHC I- and MHC II-restricted T cells. To address the importance of 4-1BB/4-1BBL interaction in an MLR for CD4 vs CD8 T cells, purified CD4 or CD8 T cells from either C57BL/6 (H-2^b) CD28$^{+/+}$ or CD28$^{-/-}$ mice were stimulated with K46J (H-2^d, B7^{low}4-1BBL^{high}) B lymphoma cells. The experiment was conducted in the presence of either 4-1BB.AP or AP control, to test the dependence of 4-1BB/4-1BBL in the MLR. In a separate experiment, addition of CTLA4.Ig to the cultures had no effect on the response, as expected from our previous studies with this cell line (10, 14, 24). We found that purified CD4 T cells from both wild-type and CD28$^{-/-}$ B6 mice exhibited a similar response to the 4-1BBL expressing APC, secreting high levels of IL-2 and IL-4 but only a small amount of IFN-γ (Fig. 4*A*). This response was blocked by soluble 4-1BB (4-1BB.AP, Fig. 4*A*). In the analogous experiment, purified CD8 T cells from wild-type or CD28$^{-/-}$ B6 mice responded with a different cytokine profile (Fig. 4*B*). The CD28$^+$ or CD28$^-$ CD8 T cells produced less IL-2 than the CD4 T cells, no detectable IL-4, and a higher level of IFN-γ than was observed in the CD4 T cell MLR. Thus, 4-1BB/4-1BBL

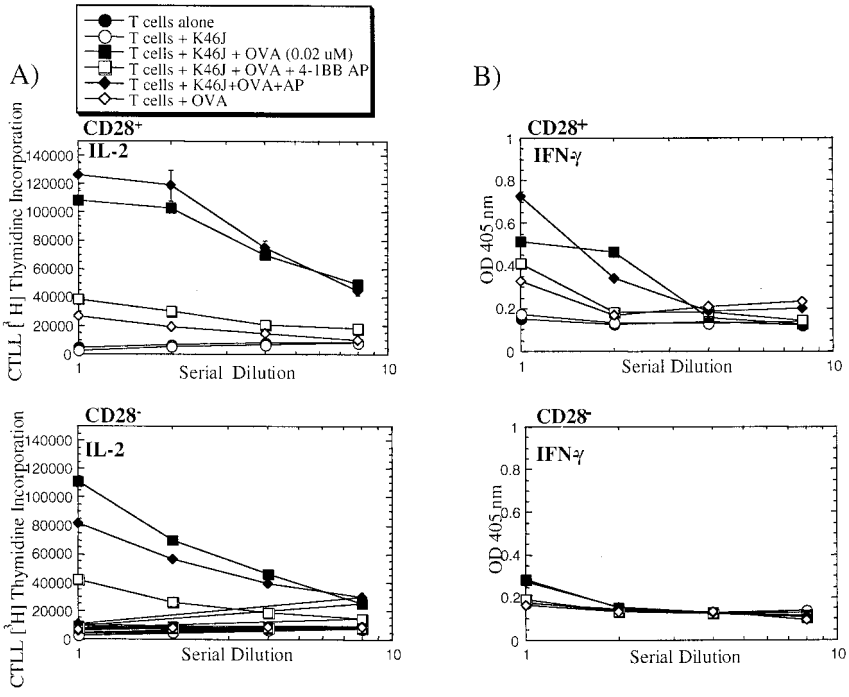

FIGURE 3. 4-1BBL augments CD4 DO-11.10 TCR transgenic T cell responses. Resting T cells (1×10^6) from DO-11.10 CD28$^+$ and DO-11.10 CD28$^-$ mice were stimulated with irradiated K46J cells (5×10^5) and 0.02 μM OVA$_{323–339}$ peptide in the presence of either 4-1BB.AP or AP control (10 μg/ml). On day 3, culture supernatant was evaluated for IL-2 (*A*) and IFN-γ (*B*) secretion. This experiment is representative of three individual experiments.

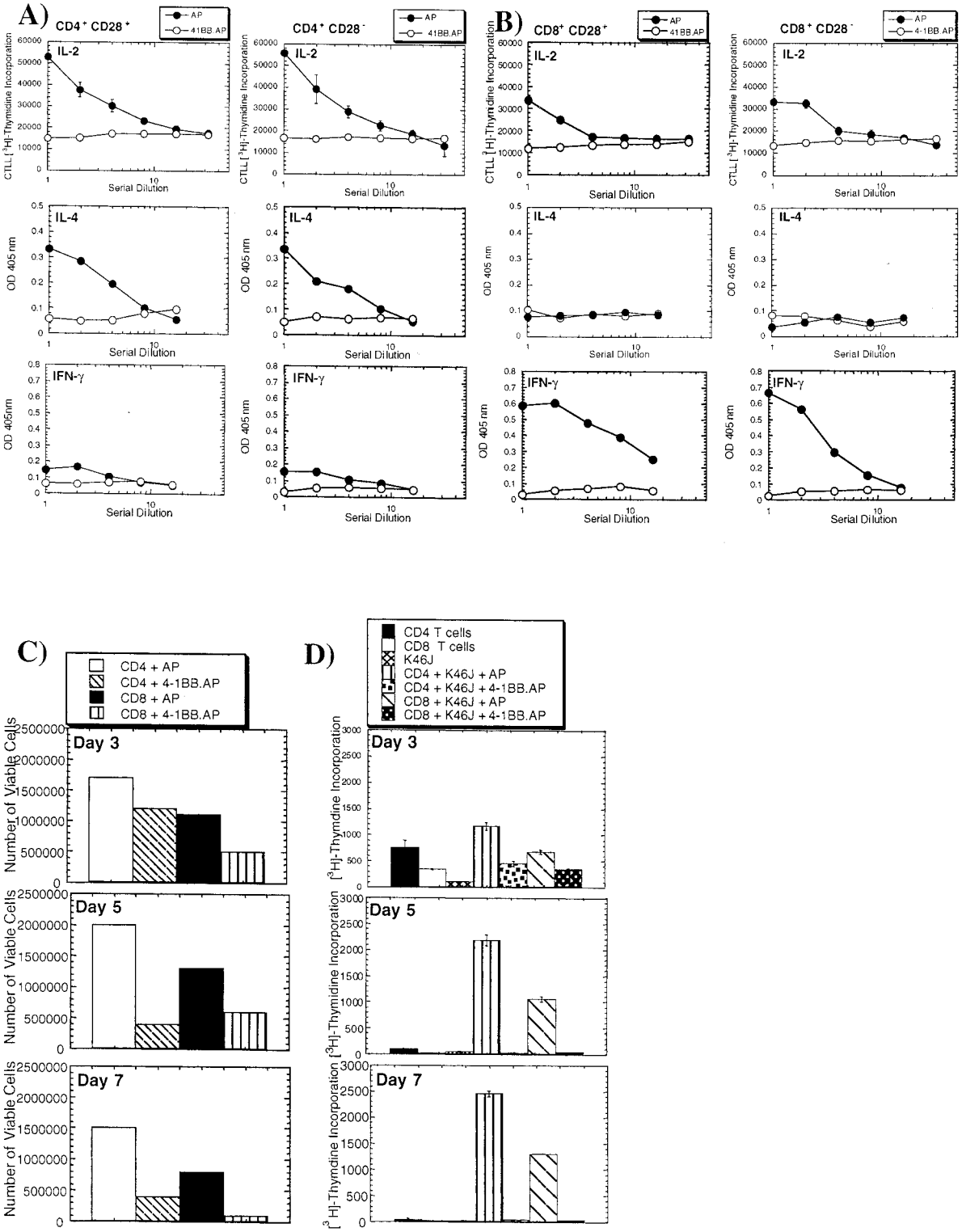

FIGURE 4. Comparison of the response of CD4 or CD8 T cells in a MLR with 4-1BBL-expressing APC. T cells were isolated from either $CD28^+$ or $CD28^-$ mice. T cells (1×10^6/ml) were stimulated with irradiated K46J cells (5×10^5/ml) in the presence of either 4-1BB.AP or AP control (10 μg/ml). *A* and *B*, On day 5 of culture, supernatant levels of IL-2, IFN-γ, and IL-4 were determined as described in *Materials and Methods*. *C*, T cell viability was assessed by counting the number of viable cells in the $CD28^+$ cultures on days 3, 5, and 7 of culture. *D*, T cell proliferation of the $CD28^+$ cultures was determined via [^{3}H]thymidine incorporation on days 3, 5, and 7 of culture. This experiment is representative of three individual experiments.

can support CD28-independent cytokine production by CD4 or CD8 T cells, albeit with a distinct cytokine profile.

To address the expansion of T cells in our assays, we counted the numbers of viable T cells at days 3, 5, and 7 of the MLR cultures (Fig. 4*C*). By day 3 of culture using 4-1BBL-expressing APC, we recovered ~1.5×10^6 viable CD4 T cells per well. This is a modest expansion over the 1×10^6 T cells seeded into the cultures. In contrast, cultures with purified CD4 T cells stimulated with another B lymphoma (M12) that expresses B7 family molecules, resulted in a 2-fold expansion of the culture by this time

point (data not shown). A comparison of T cell numbers in cultures that had been incubated in the presence of either soluble 4-1BB.AP or AP control demonstrated that in the absence of 4-1BB/4-1BBL interaction, there was a substantial decrease in T cell recovery from the cultures. The most prominent decrease in cell numbers was apparent at days 5 and 7 of culture, with a small effect at day 3. These data indicate that 4-1BB/4-1BBL interaction contributes substantially to both CD4 and CD8 T cell survival in the MLR, with larger effects later in the culture. Furthermore, there was no major difference in the sensitivity of the CD4 or CD8 T cells to 4-1BB in this assay. Fig. 4*D* compares proliferation of CD4 and CD8 T cells after stimulation with APC expressing 4-1BBL (K46J) in the presence of soluble 4-1BB.AP or with AP control. Both the CD4 and CD8 T cells exhibited 4-1BBL-dependent proliferation in the MLR, with ~2-fold greater effect of 4-1BBL on expansion of CD4 compared with CD8 T cells.

Response of CD4 vs CD8 T cells to immobilized anti-CD3 plus 4-1BBL or anti-CD28

The previous studies suggested that 4-1BBL could activate both CD4 and CD8 T cells quite effectively. However, it is conceivable that other molecules present on the APC contribute differentially to the activation of CD4 vs CD8 T cells and mask differences in CD4 vs CD8 T cell responses. Our previous results have shown that a soluble form of 4-1BBL, when immobilized on plastic with limiting amounts of anti-CD3, can augment the proliferation and cytokine production by T cells (11). Here we extend this approach to purified CD4 and CD8 T cells. Previous studies showed that optimal responses of total resting T cells to 4-1BBL occur at ~10 μg/ml of immobilized ligand coimmobilized with anti-CD3 at 1 μg/ml (11). Due to the fact that responses to 4-1BBL decline quite rapidly at lower doses of ligand, these experiments were conducted at a dose that gives maximum response. Fig. 5, *A* and *B*, demonstrated that the cytokine profiles obtained upon stimulation of CD4 and CD8 T cells with anti-CD3 plus 4-1BBL are qualitatively similar to those obtained in the MLR (Fig. 4), albeit with distinct kinetics. The CD4 T cells produced IL-2 and IL-4, whereas the CD8 T cells produced IL-2 and IFN-γ in response to 4-1BBL-mediated costimulation.

Fig. 5*C* compares the ability of immobilized anti-CD3 plus anti-CD28 vs anti-CD3 plus 4-1BBL to stimulate proliferation of CD4 and CD8 T cell responses. The concentrations of immobilized anti-CD28 and immobilized s4-1BBL were chosen so as to give a maximal T cell response when combined with anti-CD3 immobilized at 1 μg/ml (11). Shuford et al. (22) have suggested that CD4 cells respond preferentially to CD28, whereas CD8 T cells respond preferentially to 4-1BBL. However, the present results demonstrate that both T cell subsets responded to anti-CD3 plus anti-CD28 but also responded significantly to 4-1BBL-mediated costimulation. Furthermore, in this particular assay, CD4 T cells responded more vigorously than did the CD8 T cells.

High-density resting T cells show greater proliferation to anti-CD3 plus anti-CD28 than to anti-CD3 plus 4-1BBL

The results described in Fig. 5 compared 4-1BBL and CD28-induced proliferation of total T cells. This population includes resting T cells as well as T cells that may be in various stages of blastogenesis. Therefore, we isolated high density resting T cells from Percoll gradients to assess the relative effects of 4-1BBL vs anti-CD28-mediated costimulation on resting T cells. These results

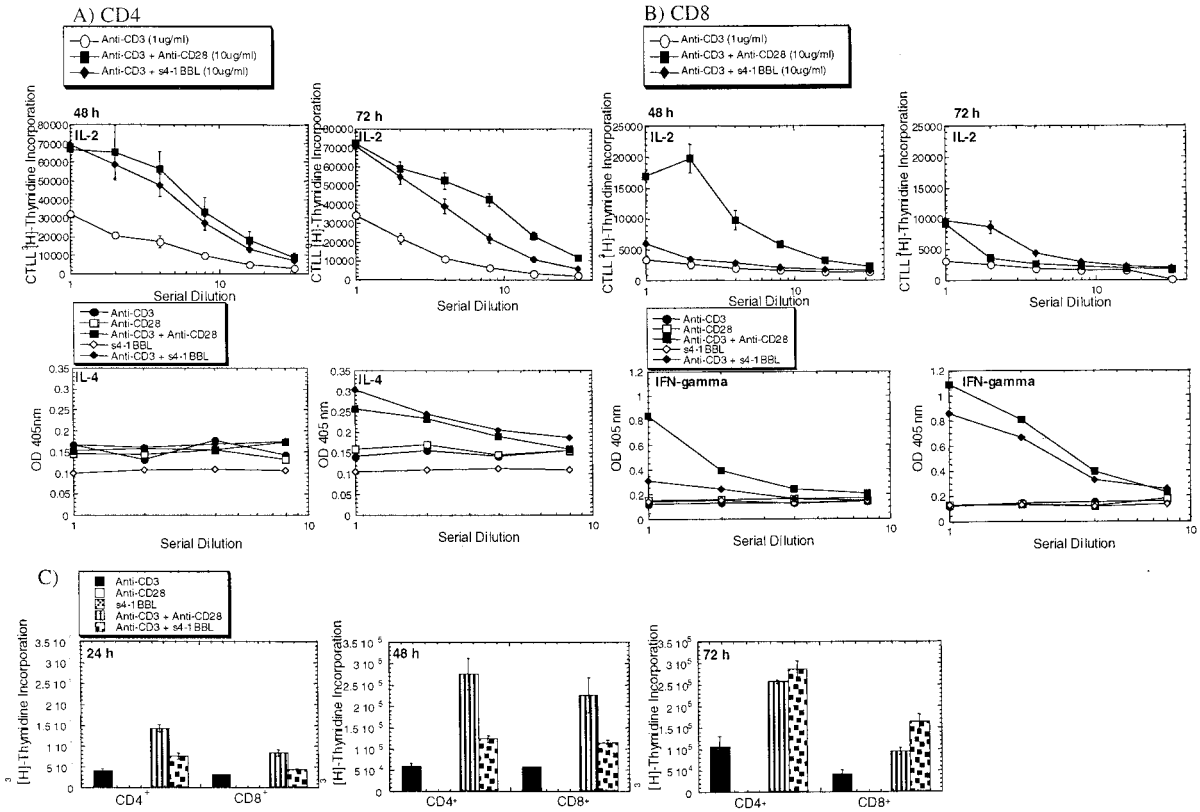

FIGURE 5. CD4 and CD8 T cell responses to 4-1BB-mediated costimulation. CD4 and CD8 T cells were purified as described in *Materials and Methods* and stimulated (1×10^6/ml) with immobilized anti-CD3 (1 μg/ml) in the absence or presence of either immobilized anti-CD28 (10 μg/ml) or immobilized s4-1BBL (10 μg/ml). *A* and *B*, Supernatant was collected at 48 and 72 h of stimulation and assessed for IL-2, IFN-γ, and IL-4 levels. *C*, T cell proliferation was assessed by [^{3}H]thymidine incorporation at 24, 48, and 72 h. This experiment is representative of three individual experiments.

illustrated a more dramatic difference in T cell proliferation when stimulated through CD28 vs 4-1BB (Fig. 6*A*) at early time points. However, at later time points this difference between CD28 and 4-1BB-mediated costimulation disappears (Fig. 6*A*, 72 h). This is consistent with the fact that resting T cells do not express 4-1BB and therefore would be delayed in their response to 4-1BB as compared with CD28.

To further analyze this effect, we used the vital dye CFSE to stain the high density resting T cells and performed flow cytometry, gating on the CD4 vs CD8 T populations, to analyze the rate of cell division. CFSE binds to intracellular proteins and is partitioned to daughter cells with each cell division (36, 39). Analysis of CFSE profiles for both CD4 and CD8 T cells indicated that naive T cells have a delay of 24 h (data not shown) between stimulation and the first round of cell division, as was also observed by Jelley-Gibbs et al. (40). After 48 h, dividing CD4 and CD8 cells are readily detected. Although CD28 costimulation gave a stronger signal, inducing a greater number of cell divisions particularly at 48 h, by 72 h there is less difference between anti-CD3 plus anti-CD28- vs anti-CD3 plus 4-1BBL-mediated stimulation. Furthermore, CD4 and CD8 showed comparable rates of cell division to both anti-CD3 plus anti-CD28 as well as anti-CD3 plus 4-1BBL.

Comparison of immobilized 4-1BBL with immobilized anti-4-1BB Ab (3H3) for stimulation of CD4 vs CD8 T cells

As mentioned above, previous reports demonstrated that CD8 cells respond better to agonistic anti-4-1BB Abs than do CD4 T cells (22, 23). One of these stimulatory anti-4-1BB Abs, 3H3, was obtained from Dr. R. Mittler (Emory University) and compared with 4-1BBL for its ability to costimulate T cell responses when immobilized together with anti-CD3 (Fig. 7). Titration experiments indicated that the concentrations of anti-4-1BB and 4-1BBL (10 μg/ml) are saturating under these conditions (data not shown). Fig. 7*A* shows the proliferative response of the T cells after stimulation for 48 h and incubation with [^{3}H]thymidine for a further 16 h. At this time point it can be observed that 4-1BBL again induced similar levels of proliferation of CD4 and CD8 T cells, whereas the

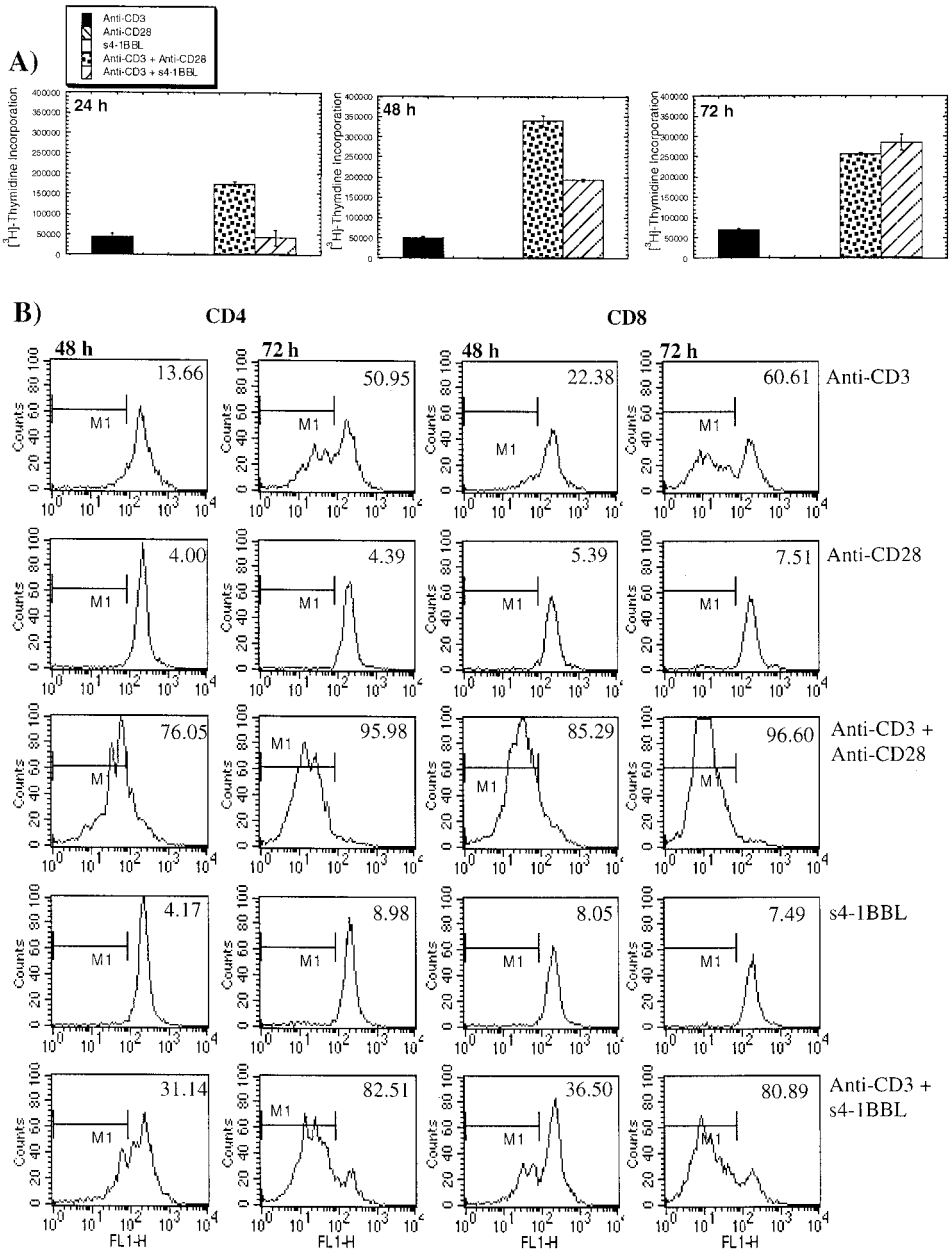

FIGURE 6. T cell proliferation in response to CD28- or 4-1BBL-mediated costimulation. High density resting T cells were isolated as described in *Materials and Methods*. T cells were stimulated with immobilized anti-CD3 (1 μg/ml) in the presence or absence of either immobilized anti-CD28 (10 μg/ml) or immobilized s4-1BBL (10 μg/ml). *A*, T cells were assessed for [^{3}H]thymidine incorporation at 24, 48, and 72 h. *B*, CD4 or CD8 cells were gated on, and CFSE dye loss was evaluated at 48 and 72 h. The numbers above each histogram indicate the percentage of dividing cells. This experiment is representative of three individual experiments.

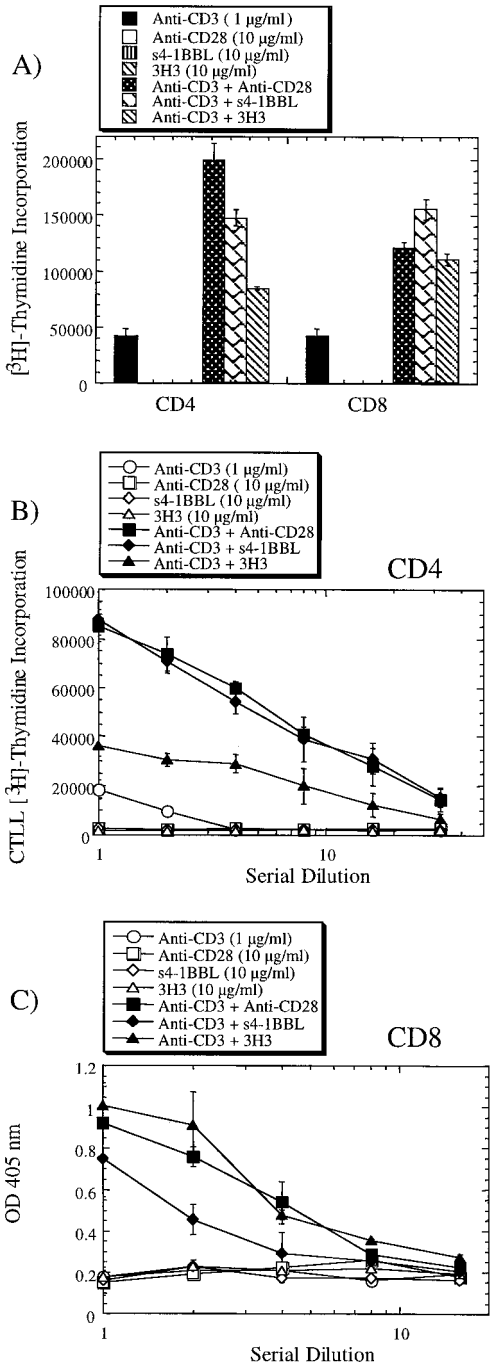

FIGURE 7. 4-1BBL- vs anti-4-1BB (3H3)-mediated costimulation. CD4 and CD8 T cells were purified as described in *Materials and Methods* and stimulated (1×10^6/ml) with immobilized anti-CD3 (1 μg/ml) in the absence or presence of immobilized anti-CD28 (10 μg/ml), immobilized s4-1BBL (10 μg/ml), or immobilized anti-4-1BB (3H3) (10 μg/ml). *A*, T cell proliferation was assessed by [^{3}H]thymidine incorporation at 48 h overnight. Supernatant was collected at 48 h of stimulation. *B*, CD4 T cells were assessed for IL-2 secretion. *C*, CD8 T cells were assessed for IFN-γ levels. This experiment is representative of two individual experiments.

3H3 Ab induced slightly higher level of proliferation of the CD8 T cells compared with the CD4 T cells (reproducible in two experiments). Following 48 h of stimulation, the CD4 T cells produce primarily IL-2, whereas the CD8 T cells produce primarily IFN-γ. Therefore, we compared 3H3 Ab with 4-1BBL for stimulation of IL-2 production by the CD4 T cells and IFN-γ production by CD8 T cells at 48 h (Fig. 7, *B* and *C*). The anti-4-1BB Ab, 3H3, was more potent than 4-1BBL in stimulating IFN-γ production by the CD8 T cells, but less potent than 4-1BBL in stimulating IL-2 production by the CD4 T cells. Thus, under the same culture conditions, 4-1BBL and 3H3 Ab appear to behave differently with respect to their effects on CD4 and CD8 T cells.

Discussion

The results presented in this study demonstrate that both CD4 and CD8 T cells can respond to 4-1BBL-mediated costimulation in a primary response to peptide Ag (Figs. 2 and 3) in a mixed lymphocyte culture (Fig. 4) and using immobilized anti-CD3 plus s4-1BBL (Figs. 5 and 6). These results indicate that 4-1BB ligation enhances T cell expansion as well as augmenting T cell effector function, including cytokine release and development of cytolytic function. These effects of 4-1BBL are independent of CD28, as both $CD28^{+/+}$ and $CD28^{-/-}$ CD4 and CD8 T cells were shown to respond to 4-1BBL-mediated costimulation. CFSE staining of primary resting T cells indicated that there was a slight delay in cell division in response to 4-1BB/4-1BBL interaction compared with anti-CD28-mediated costimulation (Fig. 6*B*). This result is consistent with the constitutive expression of CD28 and the requirement for 4-1BB induction. Analysis of T cell numbers over time in the MLR culture in the presence or absence of 4-1BB-mediated costimulation demonstrated that 4-1BB influenced cell survival, particularly at days 5–7 of culture with similar effects on both CD4 and CD8 T cells. Furthermore, analysis of cell division using CFSE staining indicated that CD4 and CD8 T cells exhibit a comparable rate of division in response to anti-CD3 plus 4-1BBL. Taken together, these results indicate that 4-1BB/4-1BBL interaction augments CD4 and CD8 T cell survival, expansion, and development of effector function subsequent to initial T cell activation.

For the P14 TCR transgenic T cells, the effects of 4-1BBL on effector function appear to be more substantial than its effects on proliferation. This finding is consistent with the recent findings of Laouar and Cripse (41), who demonstrated that proliferation and effector function of CD4 T cells are independently regulated. Furthermore, Ben-Sasson et al. (42) demonstrated that CD4 T cell division is not required for the initiation of cytokine secretion. The results presented in this report do not indicate that 4-1BBL influences the synthesis of particular cytokines, but rather that 4-1BB appears to influence the overall levels of cytokine secretion. For example, in the experiments involving the DO-11.10 TCR transgenic CD4 T cells, 4-1BBL augmented IL-2 and IFN-γ production by day 3 of culture. In contrast, when the same APC, K46J B lymphomas, were used in an MLR, the CD4 T cells produced very little IFN-γ even after 5 days of culture. Thus, it appears that other factors contribute to any cytokine skewing observed.

These results extend an earlier study by Chu et al. (10), which demonstrated that 4-1BBL could augment IL-2 and IL-4 production by purified CD4 T cells. The present study extends these results by including CD8 T cells in the analysis and adds further information regarding the survival and rate of cell division induced by 4-1BBL. Our results are also consistent with the results of Hurtado et al. (15), who showed that anti-4-1BB can inhibit activation-induced cell death induced upon reactivation of total splenic T cells with anti-CD3. In contrast, an in vivo study by Takahashi et al. (23) using anti-4-1BB Abs showed that anti-4-1BB had a much greater effect on preventing superantigen-induced cell death of CD8 T cells compared with CD4 T cells. As will be discussed below, this discrepancy may reflect the use of particular Abs against 4-1BB.

Gramaglia et al. (13) examined the effect of 4-1BBL-transfected fibroblasts on the Ag-specific response of PCC-specific TCR transgenic CD4 T cells. 4-1BBL was found to have a modest effect on enhancing IL-2 production and proliferation of primary CD4 T cells. However, 4-1BBL had little effect on short term effector cells but induced greater numbers of cells to enter cell cycle (13). The latter experiments, which used propidium iodine staining of CD4 T cells at 72 h to measure cell cycle entry, are consistent with our results using CFSE staining of CD4 and CD8 T cells.

Our observations that 4-1BBL, either expressed on APC or immobilized on plastic, induced quantitatively similar responses in CD4 and CD8 T cells in terms of proliferation, survival, cell division, and cytokine secretion, appear to conflict with the conclusions of Shuford et al. (22) and Takahashi et al. (23). These investigators concluded that CD8 T cells are much more responsive to anti-4-1BB Abs than CD4 T cells in terms of both cytokine production and proliferation. The apparent discrepancy between our results and those of Shuford et al. may be due to differences in the ability of these particular Abs to activate CD4 vs CD8 T cells rather than a difference in the function of 4-1BB on CD4 and CD8 T cells. As clearly demonstrated here, such differences are not observed with 4-1BBL-mediated stimulation.

In Fig. 7 we compared stimulatory anti-4-1BB Ab (3H3) with 4-1BBL for costimulation of CD4 and CD8 T cell responses. We found that anti-CD3 plus 3H3 (anti-4-1BB) induced slightly greater proliferation of CD8 T cells than CD4 T cells, although the magnitude of the difference was not nearly as large as reported by Shuford et al. (22). This group had reported that anti-4-1BB could augment T cell proliferation to anti-CD3 by as much as 100-fold, with only a 3- to 4-fold effect on CD4 T cells (22). In our studies, we observe smaller effects of 4-1BBL on proliferation, ranging from 2- to 5-fold depending on the specific experiment (Figs. 3–6). The smaller effects of 4-1BBL or anti-4-1BB that we observed in Fig. 7, compared with Shuford et al., may be due to the differences in experimental design. We immobilized reagents directly on plastic, whereas Shuford et al. used 0.5% APC to present the Abs via FcRs. Both anti-CD3 and 4-1BB presented by FcRs on APC may synergize with other molecules presented on the APC, leading to higher signal than observed with reagents immobilized on plastic. However, even with these differences in experimental design, we found that 4-1BBL was better than 3H3 Ab in stimulating IL-2 production by CD4 T cells but less effective than 3H3 Ab in stimulating IFN-γ production by CD8 T cells. Thus, we have demonstrated that these reagents behave differently with respect to CD4 and CD8 T cell activation. Furthermore, our results confirm the results of Shuford et al. indicating that the 3H3 Ab is particularly potent in inducing IFN-γ production by CD8 T cells. Thus, the differences observed between the anti-4-1BB Ab (3H3) and 4-1BBL in stimulating CD4 vs CD8 T cells appear to reflect a reproducible difference in the effects of these reagents on CD4 vs CD8 T cells, not explained by differences in T cell activation conditions between laboratories.

Recently, Blazar et al. (43) have come to a similar conclusion about the role of 4-1BB and its ligand in CD4 and CD8 T cell responses. Blazar et al. used purified CD4 or CD8 T cells to induce graft-versus-host disease (GVHD) in MHC-II or MHC-I mismatched recipients. By using T cells from 4-1BB$^{-/-}$ mice or 4-1BBL$^{-/-}$ recipients as well as stimulation of GVHD with anti-4-1BB Abs, these authors found that both CD4- and CD8-mediated GVHD were influenced by 4-1BB/4-1BBL to a similar extent. These data argue that CD4 and CD8 T cells respond similarly to the natural ligand of 4-1BB.

In the present study we found that both CD4 and CD8 T cells up-regulate 4-1BB during a primary response to Ag/MHC. The kinetics of the induction of 4-1BB on isolated CD4 and CD8 T cells are similar to that observed by Vinay and Kwon (3), using anti-CD3 stimulation of total splenocytes. Vinay and Kwon found peak expression of 4-1BB on unfractionated T cells between 40 and 64 h, with a decline by 110 h. In our study, CD8 P14 TCR transgenic T cells exhibited a more transient up-regulation of 4-1BB than did CD4 DO-11.10 T cells, which showed sustained 4-1BB expression up to 96 h. Gramaglia et al. (13) have analyzed the kinetics of 4-1BB expression on another CD4 TCR transgenic T cell line, the PCC-specific AND mouse. They observed peak 4-1BB expression by 48–72 h after primary stimulation, with no detectable expression by 96 h, and restimulation of the AND T cells resulted in slightly faster kinetics of 4-1BB induction. Thus, it appears that the kinetics of expression of 4-1BB during the primary response to peptide may depend more on the kinetics of the particular response being measured rather than whether the T cells are CD4 or CD8 T cells. Takahashi et al. measured the appearance of 4-1BB on CD4 and CD8 T cells during the in vivo response to superantigen. They also found 4-1BB expression on CD4 and CD8 T cells, although the expression was more rapid and transient than that observed by others in vitro. In contrast to our in vivo study, they found that the CD8 T cells up-regulated 4-1BB earlier than the CD4 T cells (23).

Our studies on the expression of TNFR family members during the primary response to Ag demonstrate that both CD4 and CD8 T cells can express 4-1BB, OX40, and CD27, in agreement with studies of these molecules in isolation (reviewed in Refs. 3–5). For the CD4 cells, we found that by 96 h, at least 96% of the T cells express all three members of the TNFR family, implying that a single cell expresses all three TNFR family members simultaneously. This raises the question of whether the signaling events induced by these molecules are redundant during the later stages of T cell activation or whether each mediates a distinct function. Recent studies using knockout mice of the CD27, 4-1BBL, OX40, or OX40L molecules have shown that removing any of these molecules alone impacts on particular immune responses. This implies that these molecules do not have redundant functions during particular immune responses in vivo (12, 19, 20, 44–48).

CD27$^{-/-}$ mice exhibit a defect in the immune response to influenza virus with fewer T cells infiltrating the lung in the CD27$^{-/-}$ mice. This effect was observed on both the CD4 and CD8 T cells. The effects of CD27 appeared to be greater during the recall response and were primarily at the level of T cell numbers without any enhancement of effector function (44).

In vivo experiments using 4-1BBL$^{-/-}$ mice have revealed a role for 4-1BBL in augmenting skin allograft rejection as well as in augmenting suboptimal cytotoxic T cell responses to influenza virus and LCMV (12, 19, 20). In the case of skin allograft rejection, the importance of CD4 vs CD8 T cells was not examined. For anti-viral responses, of three viruses examined, only the response to influenza showed a strong requirement for 4-1BBL (12). However, the role of 4-1BBL in activating influenza-specific CD4 T cells was not tested in this mouse model. Other in vivo experiments that assessed CD4 vs CD8 responses used Abs that preferentially stimulate CD8 T cells. Thus, it is difficult to assess whether the greater effects on CD8 responses are unique to these reagents. Additional immune responses need to be examined in 4-1BB$^{-/-}$ and 4-1BBL$^{-/-}$ mice before conclusions about the role of this costimulatory pathway in CD4 vs CD8 T cell during the response to infection in vivo can be made.

Studies with OX40$^{-/-}$ and OX40L$^{-/-}$ mice have shown that OX40/OX40L influences CD4 T cell proliferative responses during priming, as well as cytokine production (45–48) yet does not appear to affect CD8 T cell responses to influenza virus or LCMV (47). Studies in which OX40/OX40L interactions were blocked have implicated OX40 in Th2 responses (reviewed in Ref. 4); however, a preferential effect of OX40/OX40L on Th2 responses was not born out in studies with OX40L$^{-/-}$ mice (45, 46) or by careful in vitro analysis (49). Thus, in vivo, the evidence so far suggests that OX40 and 4-1BB play complementary roles in the immune response to influenza virus. In vitro, OX40 has been shown to influence CD4 T cell expansion and to enhance Th1 and Th2 effector function by increasing the life span of T cells (49, 50), and in this regard its function seems similar to 4-1BB. However, in contrast to 4-1BB/4-1BBL, OX40/OX40L augmentation of IL-2 production requires the presence of an intact CD28-B7 costimulatory pathway (4, 49). The finding that both OX40 and 4-1BB are expressed on both CD4 and CD8 T cells raises the question of whether CD8 T cells will also show responses to OX40L. However, as mentioned above, the evidence to date has not provided evidence for a role for OX40 in CD8 T cell responses in vivo.

What is the mechanism by which 4-1BB induces cell survival and enhancement of effector function? TNFR-associated factor (TRAF)2 provides an important link between 4-1BB and downstream signaling pathways. Our previous studies have shown that TRAF2-dependent activation of the p38 mitogen-activated protein kinase pathway is required for 4-1BB-mediated cytokine production and proliferation (11, 24). Like 4-1BB, CD27 and OX40 also recruit TRAF2 (51–53), so it seems likely that some of the common effects of these TNFR family members will be explained by downstream effects of TRAF2 recruitment. After aggregation on the surface of T cells, 4-1BB, but neither OX40 nor CD27, recruits TRAF1 (11, 53, 54). TRAF1/2 heterocomplexes can recruit the cellular inhibitors of apoptosis protein 1 and 2 (55). These proteins have been shown to directly inhibit specific caspases (56) as well as to stimulate the NF-κB pathway (57). These interactions may explain the effect of 4-1BB/4-1BBL interaction on cell survival. Both OX40 and CD27, but not 4-1BB, interact with TRAF3 and TRAF5 (51–53). Functional differences in the effects of these receptors on T cell activation may reflect differences in TRAF expression, available TRAF binding sites, and affinity of the binding sites to various TRAF proteins (58).

In summary, the results presented demonstrate that both CD4 and CD8 T cells respond to 4-1BBL-mediated costimulation. 4-1BBL engagement enhanced cell survival, cell division, and effector function with similar efficacy on both subsets. Interestingly, an anti-4-1BB Ab, 3H3, previously shown to preferentially activate CD8 T cells, was found to be less efficacious than 4-1BBL in stimulating IL-2 production by CD4 T cells, but more efficacious than 4-1BBL in stimulating IFN-γ production by CD8 T cells. In vitro CD4 and CD8 T cell subsets express both OX40 and 4-1BB, and the two costimulatory pathways appear to have similar roles in sustaining T cell survival and enhancing the development of effector function. However, in vivo there appear to be differences in their effects on particular immune responses. This may reflect differences in downstream signaling events induced by OX40 and 4-1BB and/or may be due to different localization of expression of their respective ligands in vivo.

Acknowledgments

We thank Dr. Pamela Ohashi for providing P14 transgenic mice and LCMV-related peptides, and Dr. Robert Mittler for providing 3H3 Ab. We thank Dr. Pamela Ohashi and Dr. Bernadine Leung for critical reading of the manuscript.

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