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4-1BB co-stimulation enhances human CD8+ T cell priming by augmenting the proliferation and survival of effector CD8+ T cells

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Abstract

Interactions between 4-1BB and its ligand, 4-1BBL, enhance CD8+ T cell-mediated antiviral and antitumor immunity *in vivo*. However, mechanisms regulating the priming of CD8+ T cell responses by 4-1BB remain unclear, particularly in humans. The 4-1BB receptor was undetectable on naive or resting human CD8+ T cells and induced *in vitro* by TCR triggering. Naive cord blood cells were therefore primed *in vitro* against peptides or cellular antigens and then co-stimulated with 4-1BBL or agonistic antibodies. Co-stimulation enhanced effector function such as IFN-γ production and cytotoxicity by augmenting numbers of antigen-specific and effector CD8+ T cells. OKT3 responses also showed reduced cell death and revealed that the proliferation of CD8+ T cells required two independently regulated events. One, the induction of IL-2 production, could be directly triggered by 4-1BB engagement on CD8+ T cells in the absence of accessory cells. The other, expression of CD25, was induced with variable efficacy by accessory cells. Thus, suboptimal accessory cells and 4-1BB co-stimulation combined their effects to enhance IL-2 production and proliferation. Reduced apoptosis observed after co-stimulation in the presence of accessory cells correlated with increased levels of Bcl-X_L in CD8+ T cells, while Bcl-2 expression remained unchanged. Altogether, 4-1BB enhanced expansion, survival and effector functions of newly primed CD8+ T cells, acting in part directly on these cells. As 4-1BB triggering could be protracted from the TCR signal, 4-1BB agonists may function through these mechanisms to enhance or rescue suboptimal immune responses.

Introduction

Current models indicate that effective activation of naive T cells requires two signals. The first is provided by TCR interactions with antigenic peptides presented on MHC molecules (signal 1). The second signal, so-called co-stimulation, is provided through various molecules utilizing distinct mechanisms that physically reorganize the TCR complex, lower the threshold for TCR signaling, amplify T cell activation and modify the outcome of antigenic activation, preventing anergy or death (1–4). Co-stimulation controls the priming and development of primary immune responses, but is also important for the re-activation of memory T cells (5). The co-stimulatory capacity of antigen-presenting cells (APC) is therefore an important aspect of their ability to induce and to direct immune responses (6,7)

The co-stimulatory requirements of CD4+ and CD8+ T cells can be distinguished. For instance, the expansion of LCMV virus-specific CD8+ or CD4+ T cells is differentially affected in mice respectively lacking the ligand for 4-1BB or the ligand for CD40 (8). This illustrates that specific co-stimulatory signals control optimal expansion of CD8+ T cells and outlines the

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importance of 4-1BB in CD8+ T cell responses. The molecule 4-1BB was initially described in mice as a protein up-regulated during T cell activation (9). 4-1BB is an ~55-kDa homodimeric type I membrane protein belonging to the tumor necrosis factor (TNF) receptor (TNFR) superfamily. The human and murine homologues show 60% identity at the amino-acid level with conservation in the cytoplasmic domain, but a notable difference is found in the putative Lck-binding site that is altered in human 4-1BB (10–13). Its ligand, 4-1BB ligand (4-1BBL), belongs to the TNF superfamily and it is expressed primarily on APC such as B cells and dendritic cells (DC) (14). There is little homology between the mouse and human 4-1BBL; in particular, cysteine residues are not conserved in the extracellular domains of the human and murine ligands (12), suggesting that the 4-1BB–4-1BBL system may possibly play distinct roles in the immune responses of these two species.

I*n vivo* murine studies have shown the importance of 4-1BB–4-1BBL signaling in CD8+-mediated cellular immunity. Mice deficient in 4-1BBL respond suboptimally to some viral infections, producing lower numbers of virus-specific CD8+ T cells and reduced virus-specific cytotoxicity, defects further aggravated if mice also lack CD28 (8,15). Splenocytes lacking the 4-1BB receptor induce less graft versus host disease (GvHD) lethality than normal counterparts (16). Reciprocally, the *in vivo* administration of recombinant 4-1BBL or of anti-4-1BB agonistic antibodies enhances cell-mediated immunity, cytolytic T cell activation, IFN-γ production, GvHD and leads to the rejection of allografts or tumor cells (17,18). Tumor cells transfected with 4-1BBL cDNA are more immunogenic, induce the production of lymphoma-specific cytotoxic T cells and confer protection against further challenge with parental tumor cells (19). *In vivo*, both CD40 and 4-1BB signals appear to be equally efficient in enabling CTL priming against tumor cells in the absence of CD4+ T cells (20). Altogether, there is ample evidence in mice that interactions of 4-1BB with its ligand regulate CD8+ T cell-mediated immunity *in vivo* and this may involve complex cellular interactions.

The expression of 4-1BB is not restricted to CD8+ T cells as 4-1BB is also detected on activated CD4+ T cells and activated NK1.1+ cells (21,22). The mRNA for 4-1BB has also been found in Epstein–Barr virus-transformed lymphoblastic cell line (EBV-LCL) (23) and the 4-1BB protein is expressed on a subpopulation of tonsillar B cells, but not on those in the circulation (R. S. Mittler, unpublished observations). Murine CD4+ T cells can be directly and effectively co-stimulated by 4-1BB (24,25), and the lethality of CD4+ T cells in a GvHD model is enhanced by administration of anti-4-1BB antibodies (16), indicating that 4-1BB plays an important role in the activation of CD4+ T cells *in vivo*. However, purified preparations of CD8+ T cells can be co-stimulated *in vitro* in the presence of 0.5% accessory cells (17). This effect does not require CD28 (26,27), further emphasizing that distinct mechanisms regulate the optimal co-stimulation of CD8 and CD4 T cells. In addition to mitogenic effects, 4-1BB provides an *in vivo* survival signal to murine CD8+ T cells, maintaining their viability after superantigen activation (28). Yet, like other members of the TNFR family, 4-1BB appears to mediate both anti-apoptotic (26) or pro-apoptotic effects (12) and molecules involved in these pathways are not well characterized.

Fewer studies have addressed the role of 4-1BB co-stimulation in human cells, yet these are justified by differences among species at the level of receptor or ligand and are necessary to define the therapeutic potential of 4-1BB co-stimulation. It is known that antibodies and 4-1BBL-transfected cells are mitogenic to CD3-activated human blood lymphocytes (12), and in such polyclonal activation systems with adult blood cells, that 4-1BB stimulation enhances the production of IFN-γ and IL-2 in human CD4+ and CD8+ T cells (29,30), and induces the adherence of human CD4+ T cells to fibronectin (31). Our own studies have implicated 4-1BB as a molecule regulating the activation and/or expansion of peptide-specific CD8+ T cells by DC (32). However, evidence that 4-1BB regulates the peptide-specific priming of naive T cells has not been reported in human CD8+ T cells. This prompted us to ask if 4-1BB could directly regulate the priming of human naive CD8+ T cells and analyze such co-stimulatory mechanisms in naive cells. In the present study, we show that the *in vitro* priming of naive CD8+ T cells in cord blood or adult blood can be enhanced by 4-1BB stimulation via recombinant ligand or agonistic antibodies and the effect is particularly visible when suboptimal APC conditions are used. 4-1BB promotes effector CD8+ T cell expansion, directly stimulates IL-2 production and provides a survival signal involving increased Bcl-X_L levels. As 4-1BB stimulation can be protracted from the TCR signal, we speculate that 4-1BB is important for the survival of primed cells, supporting a role to enhance suboptimal immune responses.

Methods

Source of cells

Neonatal umbilical cord blood (UCB) and peripheral blood from normal donors were obtained with approval from the Institutional Review Board of Wayne State University.

Cell preparation and purification

Blood mononuclear cells (MNC) obtained by centrifugation over Ficoll (Amersham Pharmacia Biotech, Piscataway, NJ) were used after plastic adherence in complete medium for 2 h at 37°C and removal of non-adherent cells by gentle washes. Purified CD8+ T cells were prepared from non-adherent MNC using negative selection. MNC were incubated with human γ-globulins (1 mg/ml) to block non-specific Fc receptor binding, then with mAb purified from hybridomas obtained from ATCC (Manassas, VA), and specific for glycophorin A (10F7MN), CD14 (3C10-1E12), CD32 (IV3), CD11b (OKM1), CD40 (G28-5) and CD4 (OKT4). Red blood cells, phagocytes, B cells, monocytes and CD4+ T cells were then removed using magnetic beads coupled to goat anti-mouse antibodies (Dynal, Lake Success, NY). Magnetic bead selection was repeated after adding purified anti-CD20 and anti-HLA-DR antibodies (Caltag, Burlingame, CA) to further remove B cells and APC. The negative fraction routinely contained >98% CD3+ T cells and >90% CD8+ T cells.

Preparation of DC

DC were generated from monocytes and from CD34+ progenitor cells as previously described (32). Briefly, adherent

MNC were cultured for 8 days in complete medium composed of RPMI 1640 (Gibco/BRL, Life Technologies, Gaithersburg, MD) 10% FCS (Hyclone, Logan, UT), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2×10^{-5} M β-mercaptoethanol. The cytokines human recombinant (rh) granulocyte macrophage colony stimulating factor (GM-CSF) (25 ng/ml), a kind gift from Immunex (Seattle, WA) and rhIL-4 (100 U/ml), a kind gift from Dr Yssel (DNAX, Palo Alto, CA) were added to cells and medium was changed every 3 days. DC maturation was induced by adding 50 ng/ml rhTNF-α (Research Diagnostics, Flanders, NJ) to the medium for the last 3 days of culture. Progenitor-derived DC were prepared by culturing UCB CD34⁺ progenitor cells in complete medium in the presence of so-called FKGmT4 cytokines consisting of rhFlt-3 ligand and rhGM-CSF (25 ng/ml each; kind gifts of Immunex), rhc-kit ligand (25 ng/ml; kind gift of SySTEmix, Palo Alto, CA), rhTNF-α (50 ng/ml; Research Diagnostics) and rhIL-4 (100 U/ml; kind gift from Dr Yssel). Progenitor cells were cultured for 7 days and the resulting DC-containing cultures were used to prime T cell responses.

Naive T cell priming

Priming against the HLA-A2-binding influenza matrix peptide flu-MP (58–66 GILGFVTL; Genemed Synthesis, San Francisco, CA) was performed using HLA-A2⁺ cells, identified by staining with the BB7.2 antibody (ATCC). Non-adherent UCB MNC were stimulated with peptide-pulsed autologous APC at the respective ratio of 10:1 in complete medium supplemented with 10 ng/ml rhIL-7 (R & D Systems, Minneapolis, MN). APC consisted of adherent monocytes (94–97% CD14⁺), autologous monocyte-derived DC or autologous CD34⁺ cell-derived DC. Peptide was loaded onto APC at the concentration of 10 μg/ml for 2 h and then washed extensively. Priming against allogeneic EBV-LCL was performed by culturing 4×10^6 UCB MNC with an allogeneic irradiated (6000 cGy) EBV-LCL (ratio 10:1) in complete medium supplemented with 10 ng/ml rhIL-7 (R & D Systems). One day after either peptide priming or EBV-LCL priming, the entire culture was transferred into plates coated with anti-4-1BB mAb (9D6, rat IgG2a, 25 μg/ml), 4-1BBL–mCD8 fusion protein (1 μg/ml; Ancell, Bayport, MN) or irrelevant rat isotype control-coated plates. Cells were re-stimulated at day 7 under the same conditions used for priming. Fresh medium was added at day 3 or 4 following stimulation. At day 14 and beyond, cells were re-stimulated as described above, but also received 10% T cell-conditioned medium (T-Stim; Collaborative Biomedical, Becton Dickinson, Bedford, MA).

Flow cytometric analysis

Cell surface antigen detection procedures have been described elsewhere (33). Directly labeled mAb included FITC–anti-CD3, FITC–anti-CD27, FITC–anti-CD25, phycoerythrin (PE)–anti-CD1a, PE–anti-CD45RO, PE–anti-CD45RA, PE–anti-CD4, PE–anti-CD137, TriColor–anti-CD4, TriColor–anti-CD19, allophycocyanin–anti-CD8 (Caltag, Burlingame CA), FITC–anti-TCRα/β, FITC–anti-CD45RA (PharMingen, San Diego, CA) and FITC–anti-CD14 (3C10-1E12 hybridoma, conjugated in our laboratory). A2-flu-MP-specific allophycocyanin-labeled tetramers were obtained from NIAID MHC Tetramer Core Facility (Atlanta, GA). Tetramer binding was performed at 4°C during 30 min and T cell specificity was confirmed by simultaneous staining with anti-TCRαβ mAb. Data were acquired using a FACSCalibur (Becton Dickinson, San Jose, CA) and analyzed with a WinMDI software, version 2.8 (Scripps Research Institute, La Jolla, CA).

Intracellular staining for IFN-γ was performed after a short antigenic re-stimulation during 5 h at 37°C in the presence of 2 μM Monensin (Sigma, St Louis, MO). After washing, cells were stained for cell surface antigens as described above, and then fixed with 4% formaldehyde at 4°C for 5 min and permeabilized with 0.1% saponin (Sigma) in staining buffer. After washing and blocking Fc receptor binding with human γ-globulins, cells were incubated during 30 min at 4°C with PE–anti-IFN-γ mAb (PharMingen) diluted in permeabilization buffer. A positive control consisted of T cells stimulated with 50 ng/ml phorbol myristate acetate (PMA; Sigma) and 500 ng/ml calcium ionophore A23187 (Sigma). Negative controls consisted of T cells stained with irrelevant mouse IgG (Caltag).

Apoptosis was measured by exposure of phosphatidylserine at the surface of live but apoptotic cells by flow cytometry using FITC-conjugated Annexin V (Caltag) and either 7-AAD (Viaprobe; PharMingen) or Topro-3 (Molecular Probes, Eugene, OR) as vital dye. The percentage of apoptotic cells was determined in the region of cells excluding the vital dye and binding Annexin V above background. Intracellular levels of Bcl-2 and Bcl-X were determined by flow cytometry after fixation in 4% formaldehyde and permeabilization with 0.1% saponin using the PE-conjugated anti-Bcl-2 antibody (clone Bcl-2/100; PharMingen) and the purified polyclonal rabbit anti-Bcl-X (Transduction, Lexington, KY) revealed by FITC-conjugated goat anti-rabbit antibodies (Vector, Burlingame, CA). Negative controls included cells stained with irrelevant antibodies.

Polyclonal T cell proliferation

Polyclonal proliferation of purified naive CD8⁺ T cells, UCB MNC or flu-MP-specific cells (5×10^4 cells/0.2 ml of complete media per well in triplicate) was induced by culture on plastic plates coated with suboptimal (10–50 ng/ml) or optimal (1–10 μg/ml) concentrations of OKT3 mAb (Orthoclone; Ortho Diagnostics, Westwood, MA). During the last 10 h of culture, 1 μCi [^{3}H]thymidine (NEN, Boston, MA) was added to each well. Cells were harvested (harvester 96; Tomtec, Hamden, CT) and counted using a liquid scintillation counter (Trilux; Perkin-Elmer Wallac, Gaithersburg, MD). Stimulation indexes (SI) were calculated as the average proliferation of triplicate wells in test cultures divided by the average proliferation of triplicate wells in control cultures containing an irrelevant rat IgG2a, κ mAb (clone R35-95; PharMingen).

Cytokine measurements

The production of IL-2 or IFN-γ in the culture medium was measured using the OptiEIA human IL-2 kit or OptiEIA human IFN-γ kit according to manufacturer's instructions (PharMingen).

Cytotoxicity assay

Cytotoxicity was measured 14 days after priming in a standard ^{51}Cr-release assay. Targets consisted of peptide-coated ^{51}Cr-

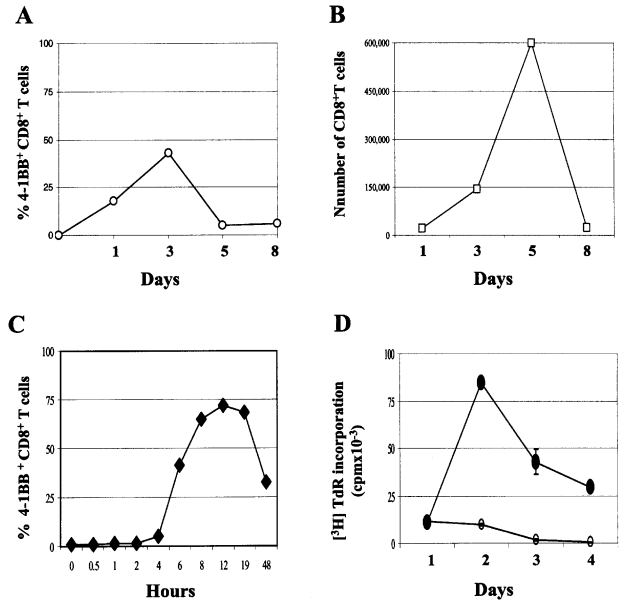

Fig. 1. Regulation of 4-1BB expression on naive and antigen-specific CD8+ T cells. Naive UCB T cells (A and B) or flu-MP-specific T cells (C and D) were analyzed. The expression of 4-1BB was measured by multi-color flow cytometry in CD8+ T cells at different time points after stimulation with an optimal concentration of OKT3 (10 μg/ml). Results are expressed as percentage of cells expressing 4-1BB within the CD8+ T cell population at different time points. Proliferation was measured at different time points by numbers of CD8+ T cells by manual cell count and flow cytometry (open squares, B) or by [3H]thymidine incorporation in flu-MP-specific T cells (filled circles, OKT3 stimulation and open circles, unstimulated control, D). Results are representative of two independent experiments.

labeled T2 cells (10 μg/ml specific or irrelevant peptide added overnight) or EBV-LCL as targets [100 μCi $Na^{51}CrO_4$ (NEN, Boston, MA) for 2 h at 37°C] and unlabeled K562 to exhaust NK/LAK activity. After four washes, 5000 labeled target cells and 5000 unlabeled K562 cells were added to lymphocytes (E:T ratio 10:1) in triplicate and incubated for 5 h at 37°C. Supernatant fluid was harvested to measure ^{51}Cr release by liquid scintillation. Spontaneous release was determined by incubation of target cells alone and maximum release was determined by incubation of target cells with 0.1 N HCl solution. The percentage of specific lysis was calculated as: 100 × [(experimental – spontaneous release)/(maximal – spontaneous release)]. Results as expressed as specific cytotoxicity minus non-specific cytotoxicity against T2 cells pulsed with an irrelevant peptide.

flu-MP-specific T cells

A culture of flu-MP-specific T cells was obtained by priming blood MNC from an HLA-A2+ adult individual with autologous mature monocyte-derived DC using a reported technique (34). After the third re-stimulation, bulk cells were expanded in the presence of 10% T-stim conditioned medium, 10 U/ml of rhIL-2 (NCI Biological Resources Branch, Frederick, MD) and either plastic-coated OKT3 (50 ng/ml) or irradiated peptide-pulsed autologous EBV-LCL. Flow cytometry analysis demonstrated 50–70% of A2-flu-MP tetramer-specific cells in these cultures. Resting flu-MP-specific cells were obtained after a period of culture of 2 days in the absence of rhIL-2 which dramatically lowered the spontaneous [3H]thymidine incorporation in these cells.

Results

Regulation of 4-1BB expression on naive and antigen-specific CD8+ T cells

Naive T cells from neonatal UCB are useful to study the molecular requirements for the priming of antigenic T cell responses in humans. In our hands, the phenotype of UCB MNC was 25 ± 15% CD4+, 8 ± 2% CD8+ of which 91 ± 8% expressed the phenotype CD45RA+CD27+CD8+ of naive CD8+ T cells, 7.7 ± 8% expressed the phenotype CD45RA+CD27–CD8+ of effector CD8+ T cells and 2 ± 1% expressed the phenotype CD45RO+CD8+ of activated/memory CD8+ T cells (n = 9), corroborating that the great majority of UCB CD8+ T cells were antigenically naive (35). We found that 4-1BB was already detectable on a minority of freshly isolated UCB cells (0.40 ± 0.45%; n = 14), and this population was 44 ± 21% CD14+, 32 ± 13 CD56+, 12 ± 10% CD19+, 10 ± 12% CD4+CD25+ and 8.76 ± 5 CD3+CD56+, suggesting that it contained monocytes, activated/regulatory T cells and B cells. Yet, the great majority (99.8%) of CD8+ CD3+ T cells did not express detectable 4-1BB levels and neither did the great majority (99%) of monocytes. The expression of 4-1BB is inducible on human T cells by phytohemagglutinin or by calcium ionophore + PMA within 1 day, peaking at ~48 h post-activation (36,37). To our knowledge, naive T cells were not specifically examined. After polyclonal OKT3 stimulation of UCB, 4-1BB expression increased on CD8+ T cells, peaking at day 3 (Fig. 1A) preceding the peak of proliferation which occurred at day 5 (Fig. 1B), and levels of 4-1BB were down-regulated afterwards. At peak time, ~43% of primary UCB CD8+ T cells expressed 4-1BB. These results were contrasted with the analysis of resting, but previously activated antigen-specific adult T cells that had undetectable levels of 4-1BB but were 70–90% CD8+ TCR αβ+, CD28–, 50–70% A2-flu-MP tetramer+, 95% CD45RO+ and 5% CD45RA+. After optimal OKT3 stimulation, 41BB increased rapidly on these cells to peak between 10 and 20 h (Fig. 1C), followed by maximal proliferation at 48 h and a decline thereafter (Fig. 1D). At peak expression, ~70% of antigen-experienced CD8+ T cells expressed 4-1BB, substantially more than naive cells. Thus, we confirm that the 4-1BB molecule is transiently expressed after TCR engagement in a precise and narrow window of time, and that the state of previous cellular activation determines the kinetics of expression. Naive cells required more time to up-regulate 4-1BB and to reach their proliferative peak than previously activated T cells. These kinetics led us to speculate that 4-1BB may be able to function at a distance of, and subsequently to, TCR engagement in naive CD8+ T cells. Indeed, 4-1BB and TCR signals could be given in a temporally discrete manner. We stimulated UCB T cells with suboptimal OKT3 and 1 day later, exposed cells to 4-1BB agonists consisting either of agonistic anti-4-1BB mAb 9D6 or 4-1BBL-mCD8 fusion protein both coated onto microtiter plastic plates. Reagent concen-

Table 1. Exogenous 4-1BB agonists improve the priming of monocyte-induced but not DC-induced flu-MP peptide-specific T cell responses

Experiment	APC type	Days	Numbers of A2-flu-MP tetramer⁺ cells		
			Control	Anti-4-1BB mAb	4-1BBL
1	DC (CD34 derived)	0	NT	–	–
		7	64	53 (0.83)	51 (0.80)
		20	1752	2357 (1.35)	1995 (1.14)
2	DC (CD34 derived)	0	8	–	–
		9	2002	1706 (0.85)	938 (0.47)
		14	9177	4575 (0.50)	4057 (0.44)
3	DC (monocyte derived)	0	10	–	–
		14	1969	NT	1784 (0.91)
		27	13334	NT	15022 (1.13)
	monocyte	14	1490	2377 (1.60)	1650 (1.11)
		27	8784	10027 (1.14)	15625 (1.78)
4	monocyte	0	0	–	–
		7	400	626 (1.57)	905 (2.26)
		14	468	400 (0.85)	1385 (2.96)

UCB MNC were primed *in vitro* with flu-MP-pulsed autologous adherent monocytes or autologous DC. One day after being primed, cells were transferred to plates coated with rat IgG2a (control), 4-1BB mAb or 4-1BBL. At the indicated time points, cells were counted and stained with A2-flu-MP tetramers. Cell numbers are normalized to 10^5 initial cell input. Numbers in parentheses indicate the fold increase numbers of A2-flu-MP-tetramer⁺ cells under 4-1BB-mediated co-stimulation (compared to control). NT, not tested.

trations were optimized in titration experiments. This protracted co-stimulation enhanced the proliferation of CD8⁺ T cells compared to control cells receiving just the primary signal (data not shown). Based on these results, 4-1BB co-stimulation was provided 1 day after antigen stimulation in experiments aimed at measuring the priming of whole cord blood cells.

Effects of 41BB co-stimulation in the priming of CD8⁺ T cell-mediated immune responses

The role of 4-1BB co-stimulation in the priming of the human CD8⁺ T cell response has not been studied extensively. Naive T cells can be effectively primed by DC that induce a vigorous CD8⁺ T cell response to the HLA-A2⁺-binding flu-MP peptide (38). Our own results implicated 4-1BB in peptide-induced CD8⁺ T cell expansion by DC (32), therefore we examined if additional 4-1BB co-stimulation could enhance flu-MP priming by DC *in vitro*. Non-adherent MNC from HLA-A2⁺ UCB samples were primed with flu-MP peptide-pulsed autologous DC in the presence of IL-7. One day later, cells were transferred onto plates coated with 9D6 anti-4-1BB mAb or 4-1BBL. Antigen-specific CD8⁺ T cells, detected by flow cytometry using A2-flu-MP tetramer, were undetectable or at very low levels at start of culture (0–0.01%), but increased over time after DC priming (Table 1). At the time points examined, there was little or no enhancing effect of 4-1BB agonists when priming was initiated by DC derived from CD34⁺ cells or from monocytes. The addition of 4-1BB agonists sometimes reduced DC priming to values as low as 44% of control, suggesting that 4-1BB may participate in cell death. In contrast, priming was enhanced by 4-1BB agonists, particularly 4-1BBL in two experiments with adherent monocytes as APC. Figure 2 illustrates one such experiment. In the presence of 4-1BBL, numbers of A2-flu-MP tetramer⁺ cells increased to be as much as 3-fold higher than control cultures. The effects of 4-1BBL were evident after just one stimulation, i.e. at day 7 as seen in Fig. 2(B), demonstrating an effect at early stages of priming. High percentages of antigen-specific CD8⁺ T cells were found in these cultures, in some cases 50% of CD8⁺ T cells, in agreement with earlier reports of a massive expansion of antigen-induced specific CD8⁺ T cells (39). Parallel measures of IFN-γ-producing CD8⁺ cells showed that functional activity appeared later, at day 14, and was dramatically increased by 4-1BBL by ~14-fold (Fig. 2B). At day 14, significantly higher specific cytotoxicity against peptide-coated target cells was detectable in cultures treated with 4-1BBL (Fig. 2C, $P < 0.05$ at 1:10 ratio) and these results were confirmed in a separate priming experiment. Increased function was also seen in cultures stimulated with 4-1BBL, as IFN-γ-producing cells that were only a fraction of antigen-specific T cells in control cultures (Fig. 2B) became practically equivalent in numbers to antigen-specific CD8⁺ T cells determined by MHC–peptide tetramers. Altogether, these results show an enhancing effect of 4-1BBL in the priming of peptide-specific CD8⁺ T cell responses via monocytes.

The effects of 4-1BB co-stimulation were also tested in the priming of UCB T cell response against an allogeneic EBV-LCL, an antigenic system involving both CD4⁺ and CD8⁺ T cell responses. Cultures treated with 4-1BBL contained higher numbers of CD8⁺ T cells (2.8-fold greater than control cultures) at day 14 (Fig. 3A). Numbers of EBV-LCL-reactive CD8⁺ T cells were quantified by flow cytometry as IFN-γ-producing cells in response to specific re-stimulation (Fig. 3A) and were increased by 4-1BB co-stimulation at day 14 (22-fold greater than control, Fig. 3B). Accordingly, cytotoxicity was significantly increased in these cultures compared to controls (Fig. 3C). In this model system, IFN-γ-producing CD4⁺ T cells were also elicited and their numbers were increased by 4-1BBL (data not shown). These results demonstrate that 4-1BB co-stimulation can enhance the priming of CD8⁺ T cells against peptides or cellular antigens. Results also illustrate

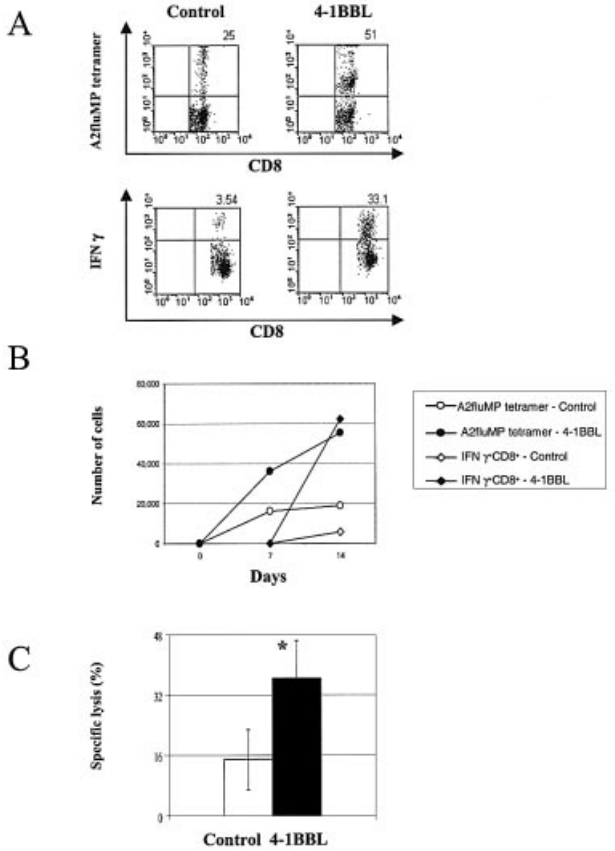

Fig. 2. 4-1BB engagement improves the priming of peptide-specific CD8⁺ T cell responses induced by monocytes. Monocytes were used to prime naive non-adherent UCB MNC against flu-MP peptide. One representative experiment out of two. (A) Two-color flow cytometric measures of A2-flu-MP-specific CD8⁺ T cells (top panel) and of intracellular IFN-γ in CD8⁺ T cells (bottom panel). A2-flu-MP-specific CD8⁺ T cells were shown to express the TCRαβ. (B) Time-dependent changes in numbers of A2-flu-MP tetramer⁺ cells (circles) and IFN-γ-producing CD8⁺ T cells (diamonds), and (C) peptide-specific killing of T2 target cells measured in the presence of 4-1BBL (black symbols) compared to control (open symbols). An asterisk indicates statistical significance ($P < 0.05$; *t*-test).

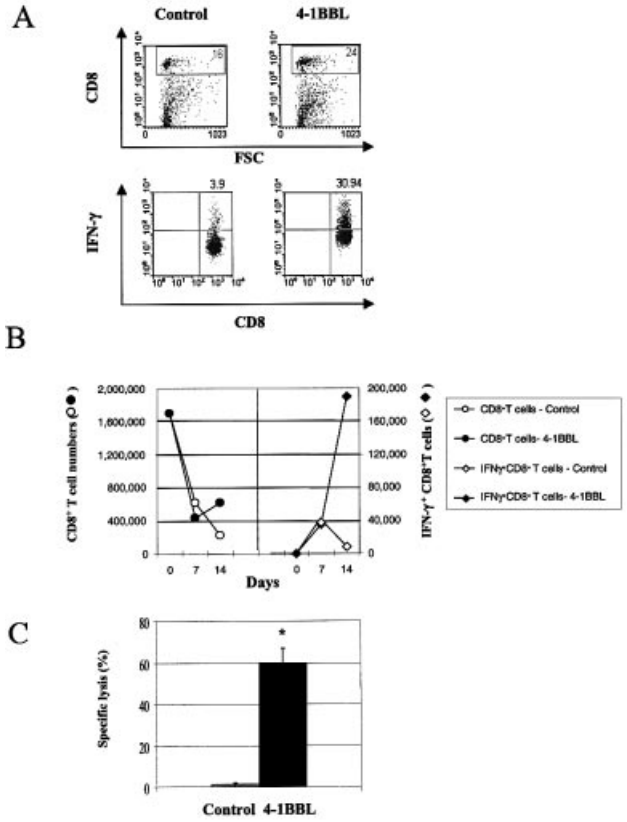

Fig. 3. 4-1BB engagement improves the priming of CD8⁺ T cell-dependent allogeneic responses induced by EBV-LCL. An EBV-LCL was used to prime the response of allogeneic naive non-adherent UCB MNC in a single experiment. (A) Two-color flow cytometric measures of CD8⁺ T cell size (top panel, percentage of CD8⁺ cells are indicated) and intracellular IFN-γ in CD8⁺ T cells (bottom panel). (B) Time-dependent changes in CD8⁺ T cell numbers (circles, left side) and in numbers of IFN-γ-producing T cells (diamonds, right side) in response to EBV-LCL stimulation in the presence (black symbols) or absence (open symbols) of 4-1BBL. (C) Increase in specific killing of EBV-LCL compared to control target cells in cultures stimulated by 4-1BBL (black bar) or control (open bar). An asterisk indicates statistical significance ($P < 0.05$; *t*-test).

that not all APC provide optimal priming and that suboptimal APC such as monocytes or EBV-LCL may be supplemented by protracted 4-1BB stimulation to provide T cell expansion comparable to that obtained after DC priming.

4-1BB co-stimulation increases numbers of effector CD8⁺ T cells

Co-stimulation via 4-1BB expanded CD8⁺ T cells prompting us to measure what subset was expanded. Prior reports had suggested that co-stimulation via 4-1BB might differentially affect subsets of CD8⁺ T cells as phytohemagglutinin-mediated polyclonal stimulation induced the 4-1BB receptor on CD45RA⁺ cells as well as on practically all transitional CD45RA⁺ CD45R0⁺ cells, but only in a fraction of memory CD45R0⁺ cells (37). MNC were polyclonally activated with OKT3 or primed with flu-MP peptide and co-stimulated via 4-1BB to measure cell surface phenotype at day 4 or 7 ($n = 3$). In all cases, 4-1BB co-stimulation increased the absolute numbers of CD8⁺ T cells after polyclonal stimulation; however, no measurable modification in CD4⁺ T cell numbers was observed (data not shown). Different subsets of CD8⁺ T cells have been identified (35). Cells with the naive CD45RA⁺CD27⁺CD8⁺ phenotype were marginally increased by co-stimulation (median 36% more cells than control cultures). Cells with the phenotype that includes memory CD8⁺ T cells (CD8⁺ CD45RO⁺ or CD45RA⁻) which are known to produce IL-2, IFN-γ and to exhibit cytotoxicity were increased by 4-1BB co-stimulation, but only up to 2-fold above control (median 58% more cells than control cultures). On the other hand, cells with the phenotype of effector CD8⁺ T cells (CD8⁺CD45RA⁺CD27⁻) which reportedly cannot secrete IL-2 but secrete IFN-γ and are dependent upon IL-2 and IL-15 for proliferation were augmented by 4-1BB co-stimulation from 2- to 8-fold above control (median 116% more cells than

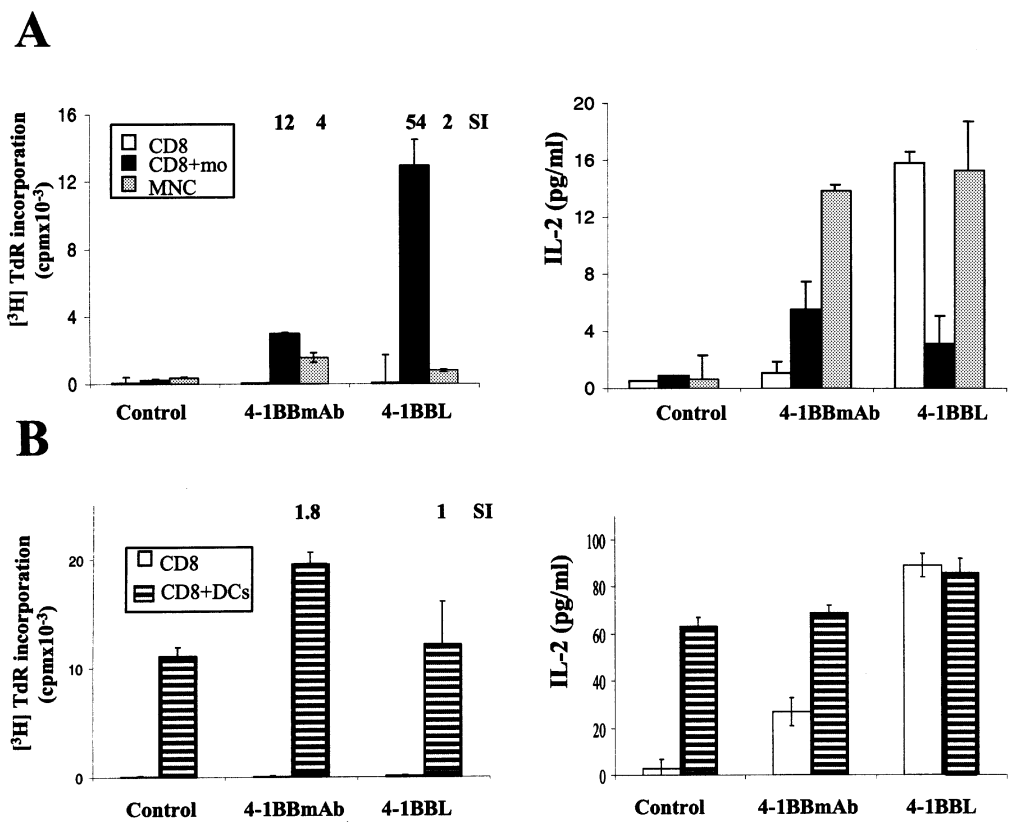

Fig. 4. Proliferation and IL-2 production in purified CD8⁺ T cells after suboptimal TCR signaling. Purified CD8⁺ T cells from adult blood donors were stimulated with suboptimal (20 ng/ml) concentrations of OKT3 bound to plastic. Two representative experiments are shown out of three. Here, 4-1BB co-stimulation was administered simultaneously with OKT3 in the presence or absence of defined populations of accessory cells at a 10% ratio. After 4 days, proliferation was analyzed by [^{3}H]thymidine incorporation and expressed as mean ± SD of triplicate wells with SI indicated on top of graphs (left panels), IL-2 secretion was measured by ELISA and expressed as mean IL-2 production ± SD of triplicate wells (right panels). (A) One experiment where pure CD8⁺ T cells were cultured with autologous irradiated monocytes or autologous MNC. (B) A separate experiment where purified CD8⁺ T cells were cultured with autologous irradiated monocyte-derived DC. Positive and negative controls for proliferation (MNC stimulated with 10 and 0 μg/ml coated OKT3) showed 27,460 ± 1,660 and 139 ± 68 for (A) and 56,027 ± 1,066 and 109 ± 30 c.p.m. for (B). Positive and negative controls for IL-2 production (same as above) were 50 ± 10 and 2 ± 3 for (A) and 109 ± 1 and 7 ± 8 pg/ml (B).

control cultures). These results confirmed that 4-1BB co-stimulation caused expansion of effector CD8⁺ T cells.

Distinct roles of 4-1BB and APC in CD8⁺ T cell co-stimulation

To understand the molecular and cellular mechanisms of CD8⁺ T cell expansion, CD8⁺ T cells from normal adult blood were stimulated with suboptimal OKT3 in the presence or absence of 4-1BB agonists and of autologous accessory cell populations. Here, for simplification of experimental design, co-stimulation was provided simultaneously with the TCR signal. In the presence of 10% irradiated autologous monocytes or 10% autologous whole MNC, CD8⁺ T cells proliferated modestly in response to OKT3 and this was enhanced by 4-1BB agonists, thus validating this experimental system (with monocytes stimulation indices of 12 and 54 in the presence of 4-1BB mAb or 4-1BBL respectively) (Fig. 4A, left panel). In the absence of accessory cells, OKT3 did not induce the proliferation of purified CD8⁺ T cells and this was not changed by addition of 4-1BB agonists (Fig. 4A and B). A separate experiment using DC as accessory cells showed that DC alone caused strong proliferative responses to OKT3 with little or no effect of 4-1BB agonists in this system (non-significant enhancements of 1.8- and 1-fold for 4-1BB mAb and 4-1BBL respectively) (Fig. 4B, left panel). The effects of autologous monocytes and DC were directly compared as shown in Fig. 5, representative of three experiments. These results confirmed the suboptimal properties of monocytes that induced less proliferation than DC on a cell-per-cell basis. Co-stimulation via 4-1BB, especially by 4-1BBL, enhanced the proliferative effects of those suboptimal APC (either monocytes or quantitatively suboptimal like 10% whole MNC), but not those of DC (Fig. 5A).

In parallel, IL-2 production was examined. IL-2 could not be induced by signal 1, in the absence of accessory cells or co-stimulation. Strikingly, the sole co-stimulation of purified CD8⁺ T cells with 4-1BB agonists directly induced IL-2 production (up to 30-fold) in the absence of accessory cells even though no proliferative effect was obtained (Fig. 4). The effects of 4-1BBL were markedly stronger than those of 4-1BB mAb and

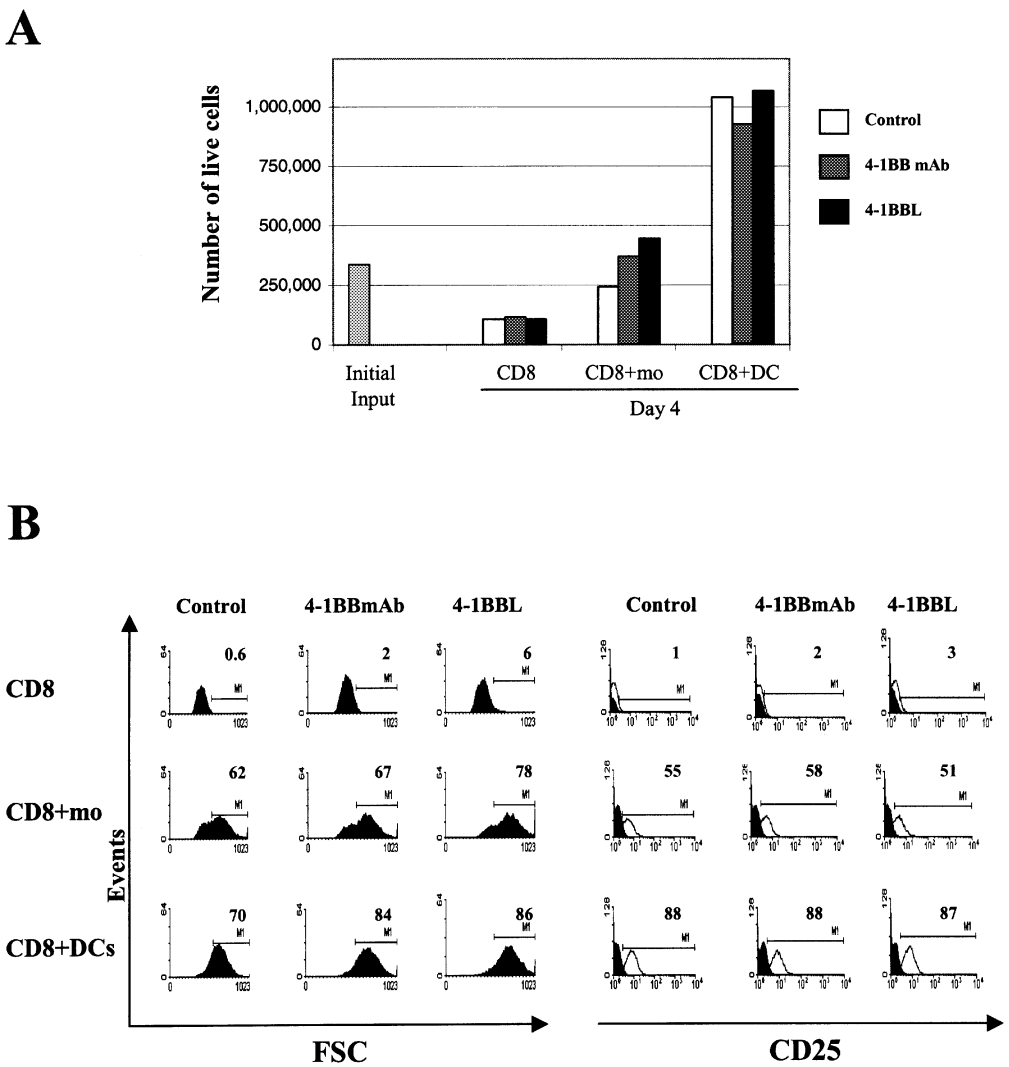

Fig. 5. Regulation of expansion, cell size and CD25 expression by APC. Purified adult blood CD8+ T cells were stimulated with suboptimal (20 ng/ml) concentrations of OKT3 bound to plastic in the presence or absence of 10% autologous monocytes or DC and in the presence of 4-1BBL (black bars), 9D6 4-1BB mAb (gray bars) and control IgG2a protein (open bars). One representative experiment out of three. After 4 days, numbers of cells were counted manually (A); CD8+ T cell size, represented by FCS, and CD25 expression were measured by flow cytometry (B). Numbers above histograms indicate the mean channel intensity of FSC or CD25 in the indicated M1 region above background.

accessory cells had no further enhancing effect. In an apparent paradox, monocytes and 4-1BBL appeared to induce lower levels of IL-2 than 4-1BBL alone or 4-1BB with MNC. Since this culture was strongly proliferative (Fig. 4A, *cf.* left and right panels) we speculated that IL-2 had been consumed by day 4. Indeed, a separate experiment where IL-2 was measured at day 2 confirmed that 4-1BBL co-stimulation in the presence of monocytes enhanced IL-2 production (data not shown). Here, 4-1BB mAb had suboptimal effects on IL-2 induction which revealed a complementary effect with accessory cells. Suboptimal APC such as monocytes which could not induce IL-2 co-operated with 4-1BB mAb to induce greater levels of IL-2 and proliferation. In contrast, with DC, 4-1BB mAb did not provide higher levels of IL-2 than those already induced by DC. These results therefore show that 4-1BB co-stimulation has a direct effect on CD8+ T cells via induction of IL-2 production. Results also show that the induction of IL-2 production is variably induced by APC, but that suboptimal conditions can be supplemented by 4-1BB agonists.

Proliferation of CD8+ T cells correlated with the appearance of large blastic cells with high FSC and with high levels of IL-2 receptor α chain (CD25) (Fig. 5B). These events were caused by accessory cells, more so by DC than monocytes, and not affected by 4-1BB agonists. Thus, the induction of CD8+ T cell proliferation after TCR engagement correlates with the induction of CD25 and IL-2 expression, which can be independently regulated events. 4-1BB co-stimulation induces IL-2 production, but not CD25, whereas accessory cells induce both events with variable efficacy, DC being the most effective. This

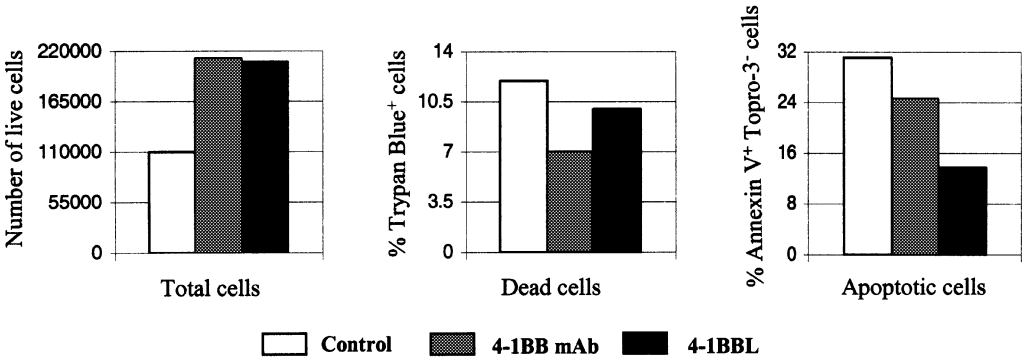

Fig. 6. 4-1BB engagement modifies T cell proliferation, apoptosis and death after suboptimal TCR engagement. UCB MNC (1.3×10^5) were activated with a suboptimal concentration of OKT3 (50 ng/ml). After 24 h, co-stimulation was performed using 9D6 4-1BB mAb (gray bars), 4-1BBL (black bars) or control IgG2a (open bars). At day 4, live and dead cells were counted using Trypan blue dye (left and middle panels). One representative experiment out of three. Apoptotic cells were assessed by flow cytometry using Annexin V binding on Topro-3 iodine-negative cells (right panel).

likely explains why 4-1BB co-stimulation enhances the proliferative effects of suboptimal APC, whereas it has no further enhancing effect in the presence of DC.

4-1BB engagement prevents apoptosis of primed T cells

In vivo experiments have shown that 4-1BB co-stimulation provides a survival signal for murine CD8⁺ T cells (28), but the molecular mechanisms resulting from 4-1BB signaling are unclear and have not been examined in human CD8⁺ T cells. In agreement with earlier reports (12), co-stimulation was apparent when total blood MNC were stimulated with suboptimal concentrations of OKT3 in the presence of anti-41BB mAb or 4-1BBL as total cell numbers increased compared to OKT3 alone (Fig. 6, left panel). As MNC proliferated, cell death also occurred. Co-stimulation via 4-1BB slightly reduced the percentages of dead MNC induced by OKT3 as measured by Trypan blue (Fig. 6, middle panel) as well as the percentage of apoptotic cells as measured by Annexin V staining by flow cytometry (Fig. 6, right panel). To analyze the mechanisms of cell survival implicated in 4-1BBL co-stimulation, we measured the expression of intracellular Bcl-2 and Bcl-X by flow cytometry 2 days after OKT3 stimulation of purified CD8⁺ T cells in the presence or absence of monocytes or DC. Undetectable in naive, unstimulated CD8⁺ T cells, Bcl-X was rapidly induced by TCR stimulation. Accessory cells, particularly DC, increased the intensity of Bcl-X expression as seen by a shift in mean fluorescence intensity of $CD8^{bright}$ cells (Fig. 7A). Adding co-stimulation further increased Bcl-X expression intensity and also increased the percentage of positive cells. In the case of co-stimulation with monocytes, higher numbers of Bcl-X⁺ cells were obtained after co-stimulation with 4-1BB mAb or 4-1BBL (Fig. 7B). Bcl-X exists as a pro-apoptotic Bcl-X_S form or as a generally more abundant anti-apoptotic form of larger size Bcl-X_L (40). Multiplex-competitive RT-PCR analysis of mRNA confirmed that Bcl-X_L was the only detectable form of Bcl-X in CD8⁺ T cells with 4-1BBL and/or APC (data not shown). RT-PCR also showed that Bcl-X_L mRNA levels, very low to undetectable in naive CD8⁺ T cells, were induced by OKT3 stimulation and did not vary with co-stimulation (data not shown), suggesting that changes in Bcl-X_L induced by 4-1BB stimulation could be post-transcriptional. In the absence of APC, a seemingly paradoxical effect was obtained as we observed that 4-1BB co-stimulation caused a reduction in Bcl-X⁺ CD8⁺ T cells (Fig. 7B). Confocal microscopic immunofluorescence analysis ruled out a change in the intracellular distribution of the protein (data not shown) and RT-PCR confirmed that Bcl-X_L was the major form, therefore suggesting that 4-1BB may have pro-apoptotic effects in purified CD8⁺ T cells in the absence of accessory cells. Presumably, and in agreement with data in Fig. 5(B), APC provide signals other than 4-1BB co-stimulation that enable the activated T cell to enter cell cycle and avoid apoptosis after TCR triggering. The anti-apoptotic molecule Bcl-2 was highly expressed on the majority of CD8⁺ T cells after OKT3 stimulation (Fig. 7B) and levels were not affected quantitatively or qualitatively by 4-1BB co-stimulation. These data agree with earlier reports showing unchanged Bcl-2 levels in murine CD8⁺ T cells activated *in vivo* by superantigen and anti 4-1BB mAb (28). Altogether, our results show that 4-1BB co-stimulation in the presence of APC protects from apoptosis and correlates with the induction of anti-apoptotic molecules such as Bcl-X_L, but not Bcl-2.

Discussion

Our study shows that 4-1BB effectively regulates the outcome of MHC class I-restricted priming, providing a survival signal to primed cells and increasing numbers of effector CD8⁺ T cells. Systems with different TCR specificity were analyzed, including a dominant viral peptide, antigens of an allogeneic EBV-LCL or polyclonal stimulation. The effects of 4-1BB were particularly evident when monocytes or B cells were used as APC suggesting that 4-1BB may rescue suboptimal antigen priming. In addition, we show that 4-1BB can function in *trans*-co-stimulatory mode and implicate Bcl-X as a target of 4-1BB-mediated effects on the survival of activated CD8⁺ T cells.

Our results demonstrate that 4-1BB co-stimulation can be provided at distance to TCR engagement and is an effective way to control the outcome of MHC class I priming. Two different mechanisms of T cell co-stimulation have been

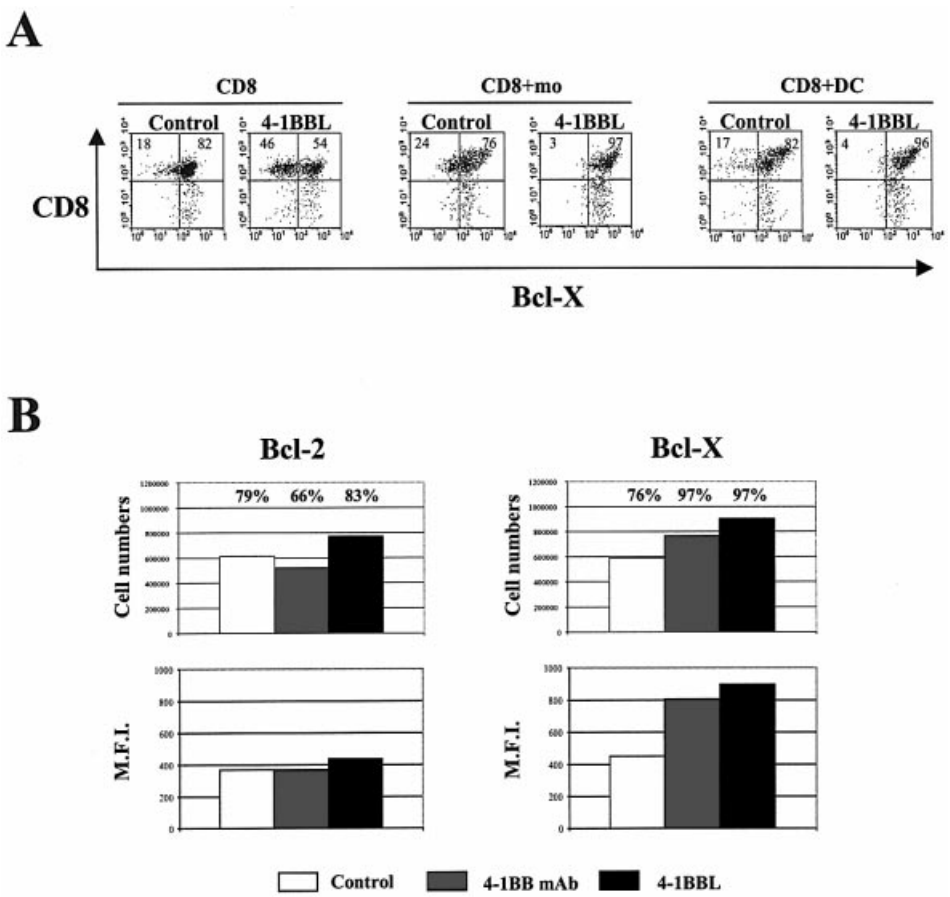

Fig. 7. 4-1BB engagement modifies the expression of Bcl-X but not Bcl-2. Data are representative of three experiments. (A) Intracellular Bcl-X expression measured 2 days after activation of purified CD8⁺ T cells with OKT3 in the presence or absence of accessory cells and 4-1BBL. Numbers indicate the percentage of positive cells within the CD8⁺ population. (B) Regulation of Bcl-2 and Bcl-X in CD8⁺ T cells co-stimulated with monocytes and either 9D6 4-1BB mAb (gray bars), 4-1BBL (black bars) or control IgG2a (open bars). Results are expressed as numbers of positive cells as measured by cell counts and flow cytometry (percentages of positive cells indicated above bars) or as the mean fluorescence intensity in the cell population expressing Bcl2 or Bcl-X above background.

proposed. *Cis*-co-stimulation delivers signal 1 and 2 simultaneously. *Trans*-co-stimulation provides signal 1 from one cell and co-stimulation possibly from another cell, presumably a mechanism to control the development of T cell differentiation into polarized effectors and the prevention of apoptosis (41–43). We show that 4-1BBL or 4-1BB mAb function as *trans*-co-stimulatory agents 1 day after signal 1. Preliminary results showed that 4-1BB agonists could provide a proliferative signal up to 4 days post-TCR engagement (data not shown), suggesting that the window of time to perform *trans*-co-stimulation may be substantial. Presentation of MHC class I determinants can occur on non-professional APC, and would result in ineffective T cell survival and expansion; therefore, our results suggest that the protracted encounter of 4-1BB ligand by antigen-primed T cells could lead to the establishment of an effective immune response. The participation of accessory signals other than 4-1BB seems indispensable since purified CD8⁺ T cells were unable to proliferate solely after TCR and 4-1BB engagement *in vitro*. Indeed, prior co-stimulatory studies were also performed in the presence of small amounts of APC (17). Our results delineate two independent steps for antigen-induced proliferation of CD8⁺ T cells. One is the induction of IL-2 production that can be triggered by 4-1BB signaling independently of accessory cells. While it had already been described that 4-1BB co-stimulation induces IL-2 production in CD4⁺ and CD8⁺ T cells (24,25,30), our results extend these observations to show that this can be a direct effect on naive CD8⁺ T cells. We also identify a step that is 4-1BB independent but accessory cell dependent, leading to response to IL-2 by up-regulation of its high-affinity receptor. Experiments using a semipermeable membrane to separate T cells and APC showed that the cell–cell contact requirement could not be substituted by CD28 stimulation (data not shown). Possibly, interactions such as LFA-1–ICAM-1, indispensable for the proliferation of purified T cells (44,45), play an important accessory role in this system. A consequence of 4-1BB co-stimulation of human CD8⁺ T cells is the control of apoptosis for the survival of newly primed cells. This appears to be mediated through the up-regulation of Bcl-X_L. These anti-apoptotic effects are consistent with prior studies in newly activated T cells (36–72 h) (26), and are reminiscent of the effects of CD28 co-stimulation that also up-regulates Bcl-X_L

and induces IL-2 (46). In addition to augmenting proliferation and survival, co-stimulation via 4-1BB enhanced the development of effector function. This agrees with a recent study showing the expansion of effector function within murine TCR transgenic CD8⁺ T cells (25). Wen *et al*. also recently reported the induction of cytolytic effector function in human T cells, which is in agreement with our findings (30). As in the murine study, we also see a more substantial effect on function than on proliferation. While it is possible that 41BB increases the functional capacity of each effector cell, our results essentially show an increase in the numbers of effector cells as measured at the individual cell level by IFN-γ production and by phenotypic markers, and could be also consistent with increased differentiation of antigen-specific cells into effector cells.

Co-stimulation was most effective with suboptimal APC, showing little enhancement of antigen-specific T cell expansion after DC priming. It is well accepted that DC are more effective APC than monocytes to present suboptimal TCR signals (47,48) and a differential requirement for 4-1BB with these two APC fits the already proposed concept that 4-1BB is more critically needed in cases of limited antigenic stimulation (15,44,49,50). Indeed, *in vivo* responses to LCMV peptides are more impaired in 4-1BBL-deficient mice than responses to whole LCMV virus infection (51). However, 4-1BB increased Bcl-X_L levels after DC priming, suggesting that 4-1BB co-stimulation may also benefit the outcome of effective antigen presentation in subtle ways. We often observed that the supplementation of monocytes with 4-1BBL appeared to equal the effects of DC. The difference between these APC, however, does not correlate with their cell surface expression of 4-1BBL. Using flow cytometry, a biotinylated 4-1BB fusion protein showed undetectable levels of 4-1BBL on monocytes as well as on the type of DC used in the present study. In contrast, EBV-LCL were readily positive (D. Laderach, unpublished observations). Possibly, 4-1BB co-stimulation may counteract negative signals provided by some APC such as monocytes or EBV-LCL which prevent the establishment of effective immune responses. An indirect effect of 4-1BB on monocytes seems unlikely as we find that very few of these cells express this receptor and these findings were confirmed by the absence of 4-1BB mRNA in monocytes by RT-PCR (23).

Our results are encouraging for the use of 4-1BB stimulation in immunotherapy, as both the recombinant ligand and the antibody show stimulatory effects. However, proliferation, IL-2 induction and survival were more effectively induced by the recombinant ligand than agonistic antibodies. Presumably, this is due to a higher avidity of the recombinant trimeric ligand for the receptor than the antibodies and may reflect integration of various molecular effector pathways downstream of the 4-1BB signal. Similarly, in mice the recombinant 4-1BBL was found to be a more effective inducer of IL-2 compared to an agonist mAb that was more effective at inducing IFN-γ (25). While we have focused on the positive aspects of 4-1BB co-stimulation, it is important to mention possible negative regulatory aspects of this molecule. Our results show that 4-1BB co-stimulation in the presence of DC has sometimes resulted in diminished numbers of cells (Table 1), suggesting that 4-1BB may possibly inhibit T cell proliferation, perhaps by augmenting death. Indeed, at late time points after DC + OKT-3 + 4-1BB stimulation we observed reduced percentages of cells expressing Bcl-X (data not shown) in a pattern similar to that seen after co-stimulation of purified CD8⁺ T cells (Fig. 7A). We speculate that in both cases, the down-regulation of Bcl-X may result from induction of high levels of IL-2, sensitizing activated T cells to AICD, as others have suggested (52,53). A negative regulatory effect of 4-1BB is consistent with pro-apoptotic effects reported in CD4⁺ T cell clones (12). A recent report that *ex vivo* activation of tumor-reactive T cells by 4-1BB mAb dampened their reactivity (54) emphasizes the subtleties of 4-1BB-mediated co-stimulation. Negative effects of 4-1BB on activated cells are likely to prevent uncontrolled expansion and activation of antigen-specific cells.

In summary, activation of naive T cells seems to be highly controlled by multiple co-stimulatory molecules encountered on the surface of the APC. We describe here that 4-1BB–4-1BBL represents a *trans*-co-stimulatory system that regulates the initiation of specific immune responses by controlling the survival, the numbers and the function of activated CD8⁺ T cells elicited after priming. Not all APC provide optimal inductive effect therefore suboptimal immune responses can be supplemented with agonists that may be useful tools in immunotherapy aimed at enhancing CD8⁺ T cell-mediated immunity.

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Abbreviations

4-1BBL	4-1BB ligand
APC	antigen-presenting cell
DC	dendritic cell
EBV	LCL-EBV-transformed lymphoblastic lymphoid cell line
GvHD	graft versus host disease
MNC	mononuclear cell
PE	phycoerythrin
PMA	phorbol myristate acetate
rh	recombinant human
SI	stimulation index
TNF	tumor necrosis factor
TNFR	TNF receptor
UCB	umbilical cord blood

References

1 Liu, Y. and Linsley, P. 1992. Costimulation of T-cell growth. *Curr. Opin. Immunol.* 4:265.
2 Sagerstrom, C., Kerr, E., Allison, J. and Davis, M. 1993. Activation and differentiation requirements of primary T cells *in vitro*. *Proc. Natl Acad. Sci. USA* 90:8987.
3 Boise, L., Minn, A., Noel, P., June, C., Accavitti, M., Lindsten, T. and Thompson, C. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-X_L. *Immunity* 3:87.
4 Yashiro-Ohtani, Y., Zhou, X., Toyo-oka, K., Tai, X., Park, C., Hamaoka, T., Abe, R., Miyake, K. and Fujiwara, H. 2000. Non-CD28 costimulatory molecules present in T cell rafts induce T cell costimulation by enhancing the association of TCR with rafts. *J. Immunol.* 164:1251.

5 Dutton, R., Bradley, L. and Swain, S. 1998. T cell memory. *Annu. Rev. Immunol.* 16:201.
6 Johnson, J. and Jenkins, M. 1993. Accessory cell-derived signals required for T cell activation. *Immunol. Res.* 12:48.
7 Borrow, P., Tough, D. F., Eto, D., Tishon, A., Grewal, I. S., Sprent, J., Flavell, R. A. and Oldstone, M. B. 1998. CD40 ligand-mediated interactions are involved in the generation of memory $CD8^+$ cytotoxic T lymphocytes (CTL) but are not required for the maintenance of CTL memory following virus infection. *J. Virol.* 72:7440.
8 Tan, J., Whitmire, J., Ahmed, R., Pearson, T. and Larsen, C. 1999. 4-1BB ligand, a member of the TNF family, is important for the generation of antiviral CD8 T cell responses. *J. Immunol.* 163:4859.
9 Kwon, B. and Weissman, S. 1989. cDNA sequences of two inducible T-cell genes. *Proc. Natl Acad. Sci. USA* 86:1963.
10 Schwarz, H., Tuckwell, J. and Lotz, M. 1993. A receptor induced by lymphocyte activation (ILA): a new member of the human nerve-growth-factor/tumor-necrosis-factor receptor family. *Gene* 134:295.
11 Kim, Y., Pollok, K., Zhou, Z., Shaw, A., Bohlen, J., Fraser, M. and Kwon, B. 1993. Novel T cell antigen 4-1BB associates with the protein tyrosine kinase $p56^{lck1}$. *J. Immunol.* 151:1255.
12 Alderson, M., Smith, C., Tough, T., Davis-Smith, T., Armitage, R., Falk, B., Roux, E., Baker, E., Sutherland, G. and Din, W. 1994. Molecular and biological characterization of human 4-1BB and its ligand. *Eur. J. Immunol.* 24:2219.
13 Zhou, Z., Kim, S., Hurtado, J., Lee, Z. H., Kim, K. K., Pollok, K. E. and Kwon, B. S. 1995. Characterization of human homologue of 4-1BB and its ligand. *Immunol. Lett.* 45:67.
14 DeBenedette, M., Shahinian, A., Mak, T. and Watts, T. 1997. Costimulation of $CD28^-$ T lymphocytes by 4-1BB ligand. *J. Immunol.* 158:551.
15 DeBenedette, M., Wen, T., Bachmann, M., Ohashi, P., Barber, B., Stocking, K., Peschon, J. and Watts, T. 1999. Analysis of 4-1BB ligand (4-1BBL)-deficient mice and of mice lacking both 4-1BBL and CD28 reveals a role for 4-1BBL in skin allograft rejection and in the cytotoxic T cell response to influenza virus. *J. Immunol.* 163:4833.
16 Blazar, B., Kwon, B., Panoskaltsis-Mortari, A., Kwak, K., Peschon, J. and Taylor, P. 2001. Ligation of 4-1BB (CDw137) regulates graft-versus-host disease, graft-versus-leukemia, and graft rejection in allogenic bone marrow transplant recipients. *J. Immunol.* 166:3174.
17 Shuford, W., Klussman, K., Tritchler, D., Loo, D., Chalupny, J., Siadak, A., Brown, T., Emswiler, J., Raecho, H., Larsen, C., Pearson, T., Ledbetter, J., Aruffo, A. and Mittler, R. 1997. 4-1BB costimulatory signals preferentially induce $CD8^+$ T cell proliferation and lead to the amplification *in vivo* of cytotoxic T cell responses. *J. Exp. Med.* 186:47.
18 Melero, I., Shuford, W., Newby, S., Aruffo, A., Ledbetter, J., Hellstrom, K., Mittler, R. and Chen, L. 1997. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Nat. Med.* 3:682.
19 Guinn, B., DeBenedette, M., Watts, T. and Berinstein, N. 1999. 4-1BBL cooperates with B7-1 and B7-2 in converting a B cell lymphoma cell line into a long-lasting antitumor vaccine. *J. Immunol.* 162:5003.
20 Diehl, L., van Lierlo, G., de Boer, A., van der Voort, E., Fransen, M., van Bostelen, L., Krimpenfort, P., Melief, C., Mittler, R., Toes, R. and Offringa, R. 2002. *In vivo* triggering through 4-1BB enables T_h-independent priming of CTL in the presence of an intact CD28 costimulatory pathway. *J. Immunol.* 168:3755.
21 Pollok, K., Kim, Y., Zhou, Z., Hurtado, J., Kim, K., Pickard, R. and Kwon, B. 1993. Inducible T cell antigen 4-1BB. Analysis of expression and function. *J. Immunol.* 150:771.
22 Melero, I., Johnston, J., Shufford, W., Mittler, R. and Chen, L. 1998. NK1.1 cells express 4-1BB (CDw137) costimulatory molecule and are required for tumor immunity elicited by anti-4-1BB monoclonal antibodies. *Cell. Immunol.* 190:167.
23 Schwarz, H., Valbracht, J., Tuckwell, J., von Kempis, J. and Lotz, M. 1995. ILA, the human 4-1BB homologue, is inducible in lymphoid and other cell lineages. *Blood* 85:1043.
24 Gramaglia, I., Cooper, D., Miner, K., Kwon, B. and Croft, M. 2000. Co-stimulation of antigen-specific CD4 T cells by 4-1BB ligand. *Eur. J. Immunol.* 30:392.
25 Cannons, J., Lau, P., Ghumman, B., DeBenedette, M., Yagita, H., Okumura, K. and Watts, T. 2001. 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy. *J. Immunol.* 167:1313.
26 Hurtado, J., Kim, Y. and Kwon, B. 1997. Signals through 4-1BB are costimulatory to previously activated splenic T cells and inhibit activation-induced cell death. *J. Immunol.* 158:2600.
27 Saoulli, K., Lee, S., Cannons, J., Yeh, W., Santana, A., Goldstein, M., Bangia, N., DeBenedette, M., Mak, T., Choi, Y. and Watts, T. 1998. CD28-independent, TRAF2-dependent costimulation of resting T cells by 4-1BB ligand. *J. Exp. Med.* 187:1849.
28 Takahashi, C., Mittler, R. and Vella, A. 1999. Cutting edge: 4-1BB is a *bona fide* CD8 T cell survival signal. *J. Immunol.* 162:5037.
29 Kim, Y., Kim, S., Mantel, P. and Kwon, B. 1998. Human 4-1BB regulates CD28 co-stimulation to promote T_h1 cell responses. *Eur. J. Immunol.* 28:881.
30 Wen, Y., Bukczynski, J. and Watts, T. 2002. 4-1BB ligand-mediated costimulation of human T cells induces CD4 and CD8 T cell expansion, cytokine production and the development of cytolytic effector function. *J. Immunol.* 168:4897.
31 Kim, Y., Mantel, P., June, C., Kim, S. and Kwon, B. 1999. 4-1BB costimulation promotes human T cell adhesion to fibronectin. *Cell. Immunol.* 192:13.
32 Ferlazzo, G., Wesa, A., Wei, W.-Z. and Galy, A. 1999. Dendritic cells generated either from $CD34^+$ progenitor cells or from monocytes differ in their ability to activate antigen-specific $CD8^+$ T cells. *J. Immunol.* 163:3597.
33 Ferlazzo, G., Klein, J., Paliard, X., Wei, W. and Galy, A. 2000. Dendritic cells generated from $CD34^+$ progenitor cells with flt3-ligand, c-kit-ligand, GM-CSF, IL-4 and TNF-α are functional antigen-presenting cells resembling mature monocyte-derived DC. *J. Immunother.* 23:48.
34 Kawashima, I., Hudson, S. J., Tsai, V., Southwood, S., Takesako, K., Appella, E., Sette, A. and Celis, E. 1998. The multi-epitope approach for immunotherapy for cancer: Identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors. *Hum. Immunol.* 59:1.
35 Hamann, D., Baars, P., Rep, M., Hooibrink, B., Kerkhof-Garde, S., Klein, M. and van Lier, R. 1997. Phenotypic and functional separation of memory and effector human $CD8^+$ T cells. *J. Exp. Med.* 186:1407.
36 Michel, J. and Schwarz, H. 2000. Expression of soluble CD137 correlates with activation-induced cell death of lymphocytes. *Cytokine* 12:742.
37 Garni-Wagner, B., Lee, Z., Kim, Y., Wilde, C., Kang, C. and Kwon, B. 1996. 4-1BB is expressed on $CD45RA^{hi}RO^{hi}$ transitional T cell in humans. *Cell. Immunol.* 169:91.
38 Larsson, M., Messmer, D., Somersan, S., Fonteneau, J., Donahoe, S., Lee, M., Dunbar, P., Cerundolo, V., Julkunen, I., Nixon, D. and Bhardwaj, N. 2000. Requirement of mature dendritic cells for efficient activation of influenza A-specific memory $CD8^+$ T cells. *J. Immunol.* 165:1182.
39 Murali-Krishna, K., Altman, J., Suresh, M., Sourdive, D., Zajac, A., Miller, J., Slansky, J. and Ahmed, R. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 8:177.
40 Boise, L., Gonzalez-Garcia, M., Postema, C., Ding, L., Lindsten, T., Turka, L., Mao, X., Nunez, G. and Thompson, C. 1993. *bcl*-x, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 74:597.
41 Ding, L. and Shevach, E. 1994. Activation of $CD4^+$ T cells by delivery of the B7 costimulatory signal on bystander antigen-presenting cells (*trans*-costimulation). *Eur. J. Immunol.* 24:859.
42 Smythe, J., Fink, P., Logan, G., Lees, J., Rowe, P. and Alexander, I. 1999. Human fibroblasts transduced with CD80 or CD86 efficiently *trans*-costimulate $CD4^+$ and $CD8^+$ T lymphocytes in HLA-restricted reactions: implications for immune augmentation cancer therapy and autoimmunity. *J. Immunol.* 163:3239.
43 Pardigon, N., Cambouris, C., Bercovici, N., Lemaitre, F., Liblau, R.

and Kourilsky, P. 2000. Delayed and separate costimulation *in vitro* supports the evidence of a transient 'excited' state of CD8⁺ T cells during activation. *J. Immunol.* 164:4493.
44 Dubey, C., Croft, M. and Swain, S. 1996. Naive and effector CD4 T cells differ in their requirements for T cell receptor versus costimulatory signals. *J. Immunol.* 157:3280.
45 Schwartz, D., Wong, R., Chatila, T., Arnaout, A., Miller, R. and Geha, R. 1989. Proliferation of highly purified T cells in response to signaling via surface receptors requires cell–cell contact. *J. Clin. Immunol.* 9:151.
46 Boise, L., Minn, A., June, C., Lindsten, T. and Thompson, C. 1995. Growth factors can enhance lymphocyte survival without committing the cell to undergo cell division. *Proc. Natl Acad. Sci. USA* 92:5491.
47 Croft, M. 1994. Activation of naive, memory and effector T cells. *Curr. Opin. Immunol.* 6:431.
48 Inaba, K., Metlay, J., Crowley, M. and Steinman, R. 1990. Dendritic cells pulsed with protein antigens *in vitro* can prime antigen-specific, MHC-restricted T cells *in situ*. *J. Exp. Med.* 172:631.
49 Kundig, T., Shahinian, A., Kawai, K., Mittrucker, H., Sebzda, E., Bachmann, M., Mak, T. and Ohashi, P. 1996. Duration of TCR stimulation determines costimulatory requirement of T cells. *Immunity* 5:41.
50 Viola, A. and Lanzavecchia, A. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science* 273:104.
51 Tan, J., Whitmire, J., Murali-Krishna, K., Ahmed, R., Altman, J., Mittler, R., Sette, A., Pearson, T. and Larsen, C. 2000. 4-1BB costimulation is required for protective anti-viral immunity after peptide vaccination. *J. Immunol.* 164:2320.
52 Lenardo, M. J. 1991. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature* 353:858.
53 Dai, Z., Konieczny, B. and Lakkis, F. 2000. The dual role of IL-2 in the generation and maintenance of CD8⁺ memory T cells. *J. Immunol.* 165:3031.
54 Kim, J., Averbrook, B., Chambers, K., Rothchild, K., Kjaergaard, J., Papay, R. and Su, S. 2001. Divergent effects of 4-1BB antibodies on anti-tumor immunity and on tumor-reactive T-cell generation. *Cancer Res.* 61:2031.