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J C Hurtado; ... et. al

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Signals Through 4-1BB Are Costimulatory to Previously Activated Splenic T Cells and Inhibit Activation-Induced Cell Death[1]

José C. Hurtado, Young-J. Kim, and Byoung S. Kwon[2]

Previously, we and others showed that signals relayed through the murine T cell Ag 4-1BB enhance primary T cell responses, and that blocking the interaction of 4-1BB with its ligand results in decreased responses to polyclonal activators and to alloantigens. Because 4-1BB expression is induced following primary stimulation, we investigated the role of signaling through this molecule in the reactivation of proliferating T cells. To this end, preactivated, 4-1BB-expressing T cells were restimulated in the presence of plate-immobilized mAbs directed against 4-1BB or the prototypic costimulatory molecule CD28. In this work, we show that in the presence of either signal, T cells respond to TCR cross-linking with strong proliferative responses and cytokine production; moreover, our findings indicate that T cell proliferation partially correlates with surface 4-1BB expression. In addition, our results suggest that Ab-mediated costimulatory signals can act independently of potential accessory B7-CD28/CTLA-4 (cytotoxic T lymphocyte Ag-4) interactions. Importantly, the characteristic DNA fragmentation and apoptotic cell death observed after TCR re-engagement are inhibited comparably in the presence of either 4-1BB or CD28 signaling. ***The Journal of Immunology,*** **1997, 158: 2600–2609.**

T cell activation of naive T cells is triggered by interaction of the TCR with its specific peptide/MHC counter-receptor on the APC; however, it is widely accepted that additional molecular interactions are required for its completion (1, 2). As a result of some of these accessory interactions, costimulatory signals are generated that favor or enhance the outcome of activation. By themselves they have no detectable effect on T cells, but in conjunction with TCR engagement they lead to significantly enhanced responses (1). In contrast, it has been proposed that TCR cross-linking in the absence of costimulatory signals induces instead a state of unresponsiveness or anergy (3, 4).

In recent years, very compelling evidence has been presented in both in vitro and in vivo model systems that indicates that the CD28 costimulatory pathway plays an important role in T cell activation (reviewed in Refs. 4–12). Working with $\alpha\beta$ TCR transgenic mice, Sagerström et al. confirmed that naive T cells fail to secrete IL-2 following TCR engagement with specific peptide/MHC complexes, but respond well when costimulated with anti-CD28 mAbs (13). In contrast, these investigators reported that previously activated T cells respond vigorously when reactivated in the absence of accessory signals (13). Nevertheless, the attractive suggestion that previously activated or proliferating T cells do not require costimulation for reactivation (13) has not been confirmed in T cells isolated from human peripheral blood, or from peripheral lymphoid tissues of normal mice. Indeed, recent reports indicate that under certain conditions, TCR engagement leads to the activation-induced cell death (AICD)[3] of previously activated mature T cells (14–20). Remarkably, the role of CD28-mediated costimulation in reactivation, AICD, and other apoptotic processes has been the subject of recent research efforts. To date, it has been reported that signaling through CD28 can prevent in vivo anti-CD3-triggered AICD of immature thymocytes (21), in vitro anti-CD3- or Ag-triggered AICD of proliferating T cells (18, 19), in vitro AICD of HIV-1-infected $CD4^+$ T cells activated with superantigens or with pokeweed mitogen (22), apoptosis of cultured naive T cells (23, 24), and the apoptotic demise observed following γ irradiation of human T cells (25) or murine $CD4^+$ T cell clones (26). More importantly, and as previously indicated for naive T cells (4–12), it has been reported that reactivation in the presence of CD28 costimulatory signals results in strong proliferative responses and cytokine secretion (18, 19, 27).

The cDNA encoding the murine T cell Ag 4-1BB was isolated and cloned by differential screening of an activated T cell library (28). As could be predicted by the cloning strategy, 4-1BB expression was found to be restricted to activated T cells (29, 30). We and others have reported recently that interfering with the interaction of 4-1BB with its ligand results in decreased proliferative responses and cytokine secretion in anti-CD3- or Ag-stimulated T cell cultures, and reduced proliferation in mixed lymphocyte reactions (31, 32). In addition, reports from our laboratory (30) and those of others (33, 34) indicate that 4-1BB can relay costimulatory signals in mitogen-stimulated primary T cell cultures (30, 33, 34), and in lectin-driven activation of thymocytes (34). Since the expression of 4-1BB follows primary activation, we conducted a series of experiments to determine whether signals relayed through this receptor were costimulatory in cultures of previously activated

Department of Microbiology and Immunology, and Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN 46202

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[2] Address correspondence and reprint requests to Dr. Byoung S. Kwon, Department of Microbiology and Immunology, Indiana University School of Medicine, 635 Barnhill Drive, Indianapolis, IN 46202.

[3] Abbreviations used in this paper: AICD, activation-induced cell death; anti-CD3s, soluble anti-CD3; CM, complete medium; CTLA-4, cytotoxic T lymphocyte Ag-4; dUTP, deoxyuridine triphosphate; MFI, mean fluorescence intensity; PE, phycoerythrin; TCGF, T cell growth factor; TdT, terminal deoxynucleotidyl transferase.

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T cells; furthermore, we sought to establish whether such signaling could alter the outcome of AICD. To this end, we established an in vitro model system in which T cells underwent two successive rounds of activation. In a first round, T cells were activated in primary cultures to induce 4-1BB expression. In a second step, purified splenic T cells were mitogen costimulated in the presence of either anti-4-1BB or anti-CD28 mAbs. We report in this work that both 4-1BB and CD28 engagement relay strong costimulatory signals to previously activated T cells, leading to enhanced proliferative responses and cytokine secretion. Additionally, we find that, as reported for CD28 (18, 19), signaling through 4-1BB in proliferating T cells prevents the AICD observed following TCR cross-linking in the absence of other accessory signals.

Materials and Methods

Mice

Female BALB/c mice (H-2^d) older than 8 wk were obtained from Harlan Laboratories (Indianapolis, IN) and maintained in a germfree environment until ready for use.

Abs, recombinant proteins, and cell lines

Unless otherwise indicated, all of the following Abs are directed against murine Ags. The hybridoma 145-2C11 (anti-CD3) and the cytotoxic T cell line CTLL-2 were obtained from American Type Culture Collection (Rockville, MD). The hybridoma 1AH2 (anti-4-1BB) was generated in our laboratory, as reported previously (30). The hybridoma 37.51 (anti-CD28) was a kind gift from Dr. James P. Allison (Department of Molecular and Cell Biology, University of California, Berkeley, CA). Anti-CD3, anti-4-1BB, and anti-CD28 mAbs were purified from ascitic fluids through protein G columns (Pharmacia, Piscataway, NJ). Purified anti-CD8, anti-trinitrophenyl, PE anti-CD4, PE anti-CD8, PE rat IgG2a, PE rat IgG2b, purified rat IgG1, biotin-conjugated anti-hamster IgG mAbs, and biotin-conjugated anti-rat IgG1 were obtained from PharMingen (San Diego, CA). PE-conjugated streptavidin was obtained from Caltag (San Francisco, CA). Goat anti-hamster IgG and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). Anti-mouse IL-2-neutralizing mAb was obtained from Genzyme Corp. (Cambridge, MA). Purified rCTLA-4Ig was obtained from Bristol-Myers Squibb (Seattle, WA). Purified human IgG1 was obtained from Sigma Chemical Co.

Splenocyte isolation and splenic T cell purification

Splenocytes were prepared as described previously (31). Briefly, spleens were removed aseptically and teased into single-cell suspensions. RBC and polymorphonuclear leukocytes were removed by density-gradient centrifugation through Lympholyte-M (Cedarlane Laboratories, Ontario, Canada). The isolated mononuclear leukocytes were washed three times and resuspended in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, penicillin (50 U/ml), streptomycin (50 μg/ml), and 50 μM 2-ME (RPMI-CM). Activated splenic T cells generated after primary activation were purified from dead cells and debris following the same procedure.

Primary splenocyte activation

For primary activation, a number equal to 5 to 7.5 $\times$ 10^6 splenocytes was added to individual wells of flat-bottom six-well plates in a final volume of 7.5 ml of RPMI-CM. Soluble anti-CD3 mAb (anti-CD3s) was added at the beginning of the culture period at a final concentration of 0.5 μg/ml. Plates were incubated in a humidified, 5% CO_2 atmosphere, at 37°C, for various time periods. After harvesting, activated splenic T cells were recovered as indicated above (density-gradient centrifugation through Lympholyte-M).

Splenic T cell reactivation assays and proliferative responses

Unless otherwise indicated, a number equal to 5 $\times$ 10^4 of previously stimulated splenic T cells was reactivated with anti-CD3s (1 μg/ml) in the presence of a fivefold excess of goat anti-hamster IgG in a final volume of 200 μl of RPMI-CM on 96-well flat-bottom microtiter plates. Before plating, varying amounts of the indicated mAbs were added to individual wells in 100 μl of PBS, pH 7.4. Plates were incubated for 3 to 4 h in a humidified, 5% CO_2 atmosphere, at 37°C, and immediately before reactivation, wells were washed three times with 200 μl of PBS to remove unbound Abs. Plates were incubated for the indicated time periods in a humidified, 5% CO_2 atmosphere, at 37°C, and pulsed with 1 μCi of [^{3}H]TdR (ICN Biochemicals, Costa Mesa, CA) per well for the final 8 to 10 h of the culture period. Cultures were harvested, and incorporated radioactivity was quantitated by liquid scintillation counting. Results are expressed as the average $\pm$ SD of triplicate wells. Reactivation assays were repeated at least three times. The results of representative experiments are shown.

Surface protein expression

Cells were stained and analyzed on a FACScan (Becton Dickinson, San Jose, CA), as described previously (29–31). Briefly, cells were harvested and washed with cold PBS, pH 7.4, before staining. A number equal to 2.5 $\times$ 10^5 cells was resuspended in 200 μl of PBS containing 1% BSA and saturating concentrations of primary (or PE-labeled) mAbs, and incubated on ice for 30 min. After washing with PBS/1% BSA, cells were stained or further incubated on ice for 20 min in 200 μl of the corresponding PE-, or biotin-conjugated Abs diluted in PBS/1% BSA. After washing twice in PBS/1% BSA, cells were either immediately analyzed or fixed with 1% paraformaldehyde (in PBS) for analysis at a later time, except for biotinylated mAb secondary incubations. In the latter case, an additional 20-min incubation with PE-streptavidin diluted in PBS/1% BSA was performed, and cells were either immediately analyzed or fixed, as indicated. Staining experiments have been repeated at least three times. Results of single determinations (10,000 live cells analyzed) from representative experiments are shown.

DNA fragmentation and cell viability determinations

The occurrence and extent of DNA fragmentation were determined based on terminal deoxynucleotidyl transferase (TdT) labeling of DNA strand breaks with fluorescein-labeled deoxyuridine triphosphate (dUTP). An in situ cell death detection kit (Boehringer Mannheim Corp., Indianapolis, IN) was used, following the manufacturer's instructions. Briefly, a number equal to 2.5 to 5 $\times$ 10^5 cells was washed twice in PBS/1% BSA, and fixed for 30 min at room temperature in a 2% paraformaldehyde/PBS solution. After washing with PBS/1% BSA, cells were permeabilized by incubation for 2 min on ice in a 0.1% Triton-X, 0.1% sodium citrate solution. After two washes with PBS/1% BSA, permeabilized cells were incubated for 60 min with labeled dUTP in the presence or absence of TdT (control), in a humidified, 10% CO_2 incubator. After three more washes in PBS/1% BSA, cells were finally resuspended in PBS/1% BSA until analyzed.

Cell viability and number were determined by direct microscopic observation of reactivated splenic T cells stained with 0.4% trypan blue (35). In addition, forward and side scatter parameters were analyzed by flow cytometry (36) and used as indicators of viability and activation alongside DNA fragmentation determinations.

Assay for the presence of TCGF in culture supernatants

Previously activated splenic T cells were cultured as indicated above for the proliferation assays, except that culture supernatants were collected after various time periods, and stored at −70°C until assayed. Total T cell growth factor (TCGF) bioactivity was determined as described (31). Briefly, 5 $\times$ 10^3 CTLL-2 cells were cultured in a final volume of 100 μl in 96-well flat-bottom microtiter plates, in the presence or absence of serial dilutions of the collected culture supernatants. TCGF bioactivity was quantitated as IL-2 U/ml by comparison with a CTLL-2 standard growth curve established with known concentrations of murine rIL-2 (Boehringer Mannheim Corp.). The contribution of IL-2 and/or IL-4 to TCGF bioactivity was determined by addition of neutralizing anti-mouse IL-2 and/or anti-mouse IL-4 mAbs. TCGF determinations have been repeated at least three times. Results of representative experiments are presented.

Results

Generation of highly enriched T cell populations

Other investigators have reported that after 48 to 72 h of primary activation with Con A, PHA, or anti-CD3s, the surviving population of splenocytes is composed almost entirely of T cells (17, 18). To confirm that these findings applied to our model system, freshly isolated splenocytes were activated in primary cell culture with anti-CD3s. After various lengths of time, live cells were isolated through density-gradient centrifugation, and analyzed by flow cytometry to determine their composition. Figure 1 presents the flow-cytometric analysis of a representative experiment. The histogram in the *top panel* shows that after 16 h of culture, 32% of the cells are T cells; by 40 h, 64% are T cells (*middle panel*); and by 64 h, nearly 98% are T cells (*bottom panel*). In conclusion, primary

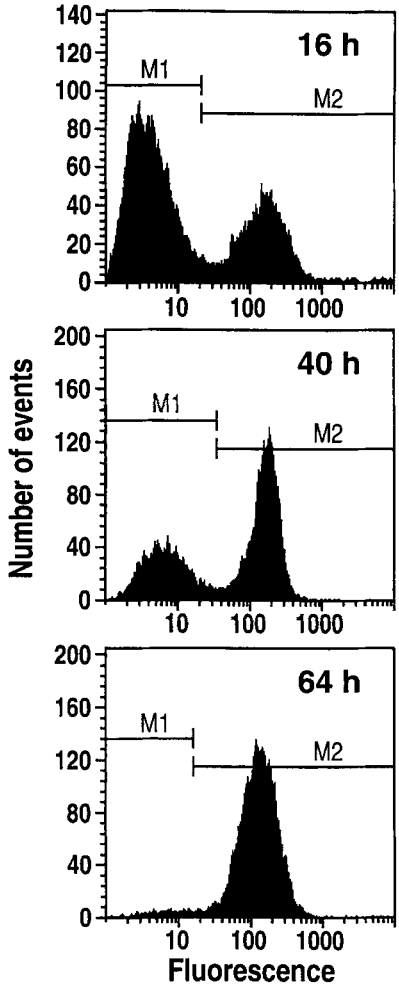

FIGURE 1. Percentage of T cells in splenocyte cultures after primary activation. After the indicated number of hours of primary activation, surviving splenocytes were isolated through density-gradient centrifugation; stained with a combination of anti-CD4/anti-CD8, or their corresponding control Abs; and analyzed by flow cytometry. Histograms corresponding to control Abs fell within the gate labeled M1 and have been omitted. Cells within the gate labeled M2 were considered positive.

activation with anti-CD3s generates a highly enriched population of splenic T cells that can be reactivated without further purification.

Expression of 4-1BB after primary activation with anti-CD3s

To optimize signaling through 4-1BB, it was necessary to determine conditions suitable for the expression of this molecule. Working with a different model system, others in our laboratory presented evidence that 4-1BB was expressed optimally at later stages of activation (29). To determine the kinetics of expression in the present model system, freshly isolated splenocytes were activated with anti-CD3s, and the expression of 4-1BB was determined by flow cytometry. Cells were removed at various time points and stained with anti-4-1BB mAb, followed by biotinylated anti-rat IgG1 mAb, and PE-streptavidin. As shown in Figure 2, after 16 h of primary activation, there is an observable (albeit minor) shift of the peak corresponding to the fluorescence intensity. 4-1BB expression is higher after 40 and 64 h (mean fluorescence intensity (MFI) of 17.1, 72.3% positive cells, and MFI of 50.6, 71.2% positive cells, respectively), and is still detectable after 5 days in culture (MFI of 4, 15.2% positive cells). Based on the kinetics of expression of 4-1BB (Fig. 2) and the time it takes to generate a highly enriched T cell population (Fig. 1), and unless otherwise indicated, 64 h was selected as a suitable time period of primary activation before reactivation in the presence of 4-1BB costimulatory signals.

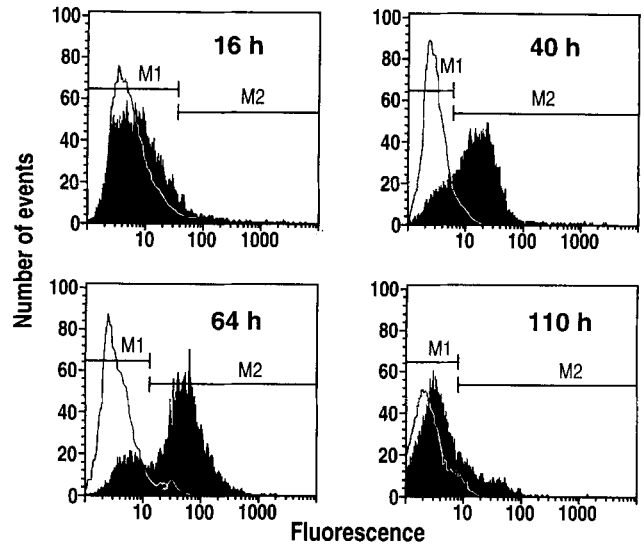

FIGURE 2. Expression of 4-1BB after primary activation. Splenocytes were activated in the presence of 0.5 μg/ml of anti-CD3s for the indicated time periods. Cells were harvested and stained in three steps with anti-4-1BB mAb, followed by biotin anti-rat IgG1, and PE-streptavidin. Flow-cytometric analysis was performed on gated live cells. Filled histograms correspond to cells stained with anti-4-1BB mAb, while empty histograms correspond to cells stained with a rat IgG1 isotype control. Cells within the gate labeled M2 were considered positive.

Splenic T cells respond to costimulatory signals provided with immobilized mAbs

As seen in Figure 3*A*, neither immobilized anti-4-1BB nor anti-CD28 mAbs had a direct stimulatory effect on previously activated splenic T cells. However, in the presence of TCR cross-linking (anti-CD3), both anti-4-1BB and anti-CD28 induced strong proliferative responses not observed in the presence of anti-CD8, anti-trinitrophenyl, or BSA (Fig. 3*A*). Moreover, preactivated splenic T cells responded to 4-1BB signals in a dose-dependent manner when reactivated with anti-CD3 in the presence of varying amounts of immobilized anti-4-1BB mAb (Fig. 3*B*). The dose-response curve indicates that 500 ng of anti-4-1BB mAb added per well is sufficient to generate a measurable lymphoproliferative response. As shown in this work, and as confirmed in similar experiments (data not shown), this concentration is nearly saturating in this model system. Based on these findings, this amount of anti-4-1BB mAb was plate immobilized in the reactivation experiments performed throughout this work. The saturating kinetics of this dose-response curve suggested that the costimulatory signals generated are specific for the anti-4-1BB mAb. We have confirmed that these signals are specific for 4-1BB (at least in part) in reactivation experiments with 4-1BB-deficient mice (unpublished results). Splenic T cells isolated from these animals failed to respond to anti-4-1BB, but responded well to anti-CD28 costimulatory signals (data not shown).

The levels of accumulated TCGF were determined in culture supernatants harvested from costimulated cultures following reactivation for various time periods. As presented in Table I, TCGF

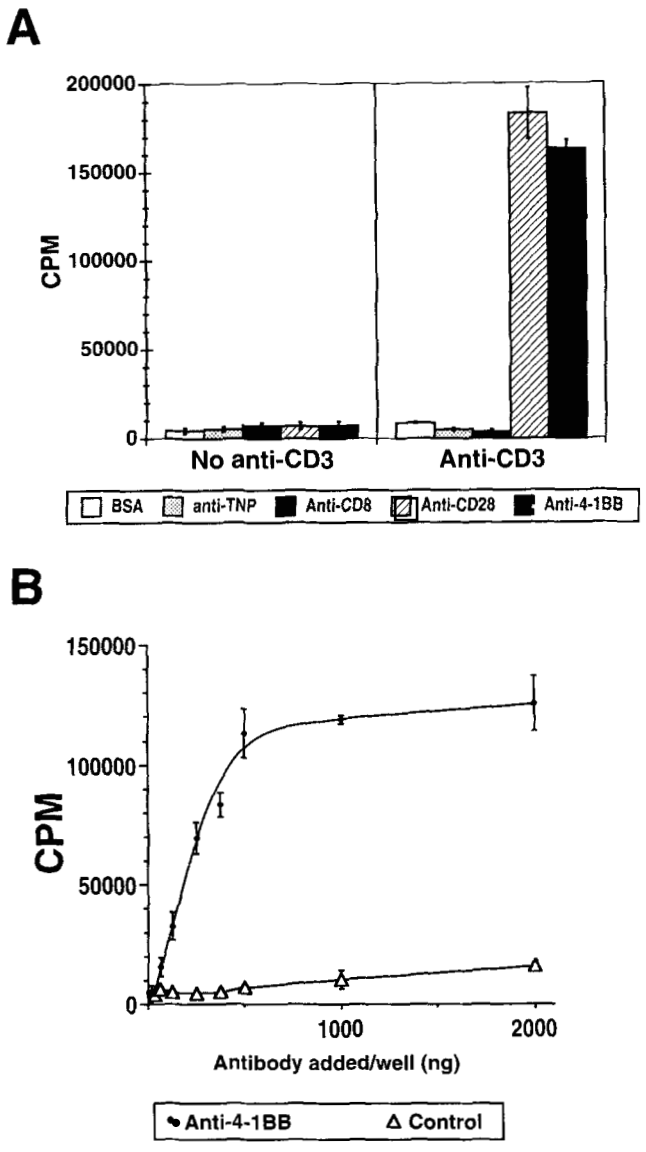

FIGURE 3. Proliferative response of previously activated splenic T cells reactivated with anti-CD3 in the presence of costimulatory signals. After 72 h of primary activation, 5×10^4 splenic T cells were reactivated with or without anti-CD3 in the presence of the indicated plate-immobilized reagents (*A*; 500 ng added/well), or in the presence of varying amounts of plate-immobilized anti-4-1BB or control (rat IgG1) mAb (*B*). Cultures were pulsed for the final 8 h of a 48-h culture period. Results are expressed as cpm. Error bars indicate SD.

bioactivity was found to be greater in costimulated cultures, and to increase as a function of time. Furthermore, bioactivity was found to be several-fold higher in the presence of CD28 costimulatory signals at the various time points. Consistent with lower TCGF production, addition of anti-IL-2 almost completely abrogated the costimulatory function of anti-4-1BB-stimulated cultures, but had no effect on anti-CD28-mediated costimulation (data not shown).

Time dependency of anti-4-1BB-mediated costimulatory signals

At various time points after primary activation, proliferating splenic T cells were isolated and reactivated with anti-CD3 in the presence of anti-4-1BB, anti-CD28, or control mAbs. Proliferative responses corresponding to four time points are presented in Figure 4. After 48 or 72 h of primary activation, the extent of the proliferative response induced with anti-4-1BB is similar to that induced with anti-CD28 mAb. This response decreases in magnitude by 96 h and is lowest by 120 h, when 4-1BB expression is still detectable, but considerably lower than at the earlier time points (Fig. 2). In contrast, the proliferative response to anti-CD28 decreases in time, but to a lesser extent: by 120 h, the response is still approximately 60% of that observed at 72 h, while the response to anti-4-1BB is less than 25% (Fig. 4).

Table I. *Anti-CD28 and anti-4-1BB cross-linking induce TCGF production by preactivated T cells* [a]

Plate-Bound Ab	IL-2 U/ml (×1000) 16 h	40 h	64 h
Anti-CD8	<0.01	<0.01	<0.01
Hamster IgG	<0.01	<0.01	<0.01
ANTI-CD28	7.9 ± 0.49	24.8 ± 0.8	37.87 ± 2.44
ANTI-4-1BB	0.31 ± 0.01	1.2 ± 0.05	1.8 ± 0.03

[a] Splenic T cells were reactivated with anti-CD3 in the presence of the indicated plate-immobilized-Abs. Supernatants were harvested at the indicated time points and assayed using CTLL-2 cells. IL-2 U/ml equivalents were quantitated by comparison with a CTLL-2 standard curve established with known concentrations of murine recombinant IL-2. Values are presented ± SD.

Anti-4-1BB-mediated signals can enhance proliferation in the absence of B7 signals

Although we indicated in Figure 1 that after 64 h of primary activation with anti-CD3s we obtain highly enriched T cell populations, it is still feasible that the remaining contaminating APC could be contributing suboptimal, but important, costimulatory signals through the interaction of B7 receptors with T cell-expressed CD28 and/or CTLA-4. Alternatively, since it has been reported in both human and murine systems that B7.1/B7.2 expression is induced or up-regulated following primary T cell activation (37, 38), it is equally likely T-T cell interactions may provide costimulatory function. In either case, anti-4-1BB mAb-generated signals might not be costimulatory by themselves, but could act in conjunction with otherwise suboptimal B7-dependent costimulation emanating from other sources. To address this issue, previously activated splenic T cells were reactivated in the additional presence of CTLA-4Ig, a chimeric molecule known to bind both B7 receptors and to effectively block B7-derived costimulatory signals (4, 5, 7, 37). As presented in Figure 5, blocking B7 interactions with CTLA-4Ig had no effect in the proliferative responses induced by either anti-4-1BB or anti-CD28 mAb signals when 25,000 or 50,000 cells were added per well (*top* and *middle panels*). However, at higher cell concentrations (*bottom panel*), CTLA-4Ig did partially inhibit [^{3}H]thymidine incorporation. These findings suggest that at higher cell concentrations (that favor either contaminating APC-T cell, or T-T cell contacts), B7 interactions can contribute to mAb-generated costimulatory signals. However, they also indicate (i.e., at lower cell numbers, such as those used throughout this work) that anti-4-1BB-mediated signals can act independently of additional B7/T cell interactions.

Anti-4-1BB-mediated signals alter the outcome of AICD

In the previous series of reactivation experiments, we had relied on [^{3}H]thymidine incorporation as a readout of the proliferative response. To confirm that proliferation was actually taking place, and to find out whether cell death was occurring in the absence of costimulatory signals, we proceeded to do direct cell-counting and viability determinations at various time points after reactivation.

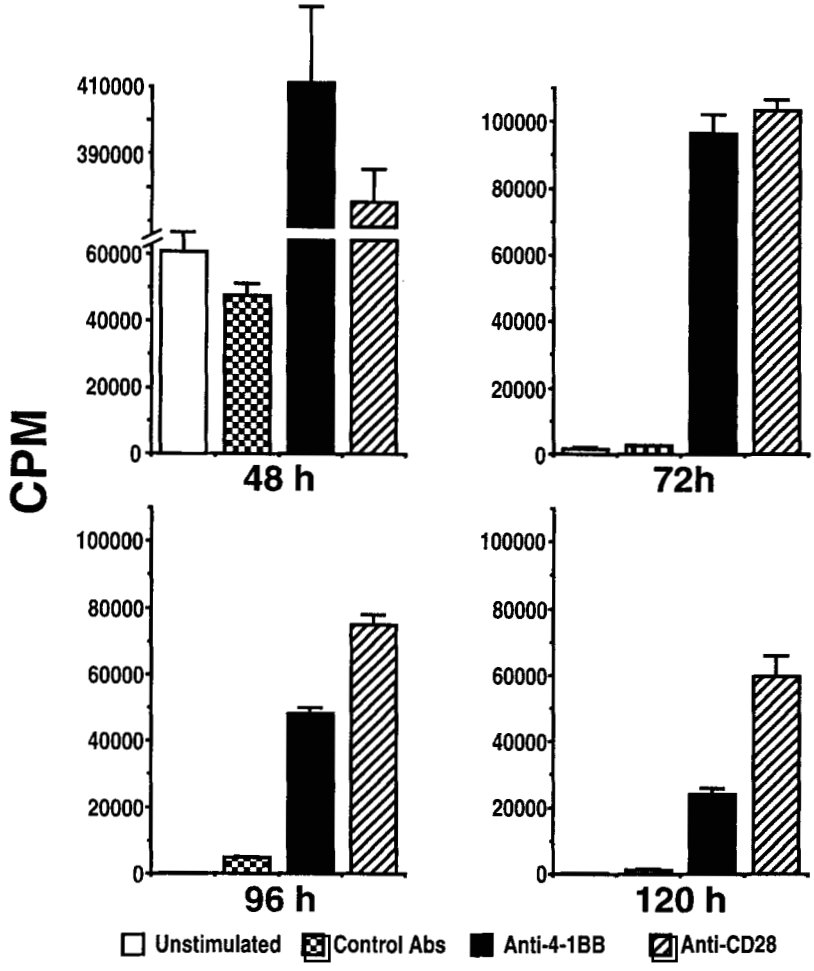

FIGURE 4. Time dependency of costimulatory function of anti-4-1BB mAb. Splenocytes were activated in primary culture for the indicated time periods. Splenic T cells were isolated and cultured in the absence (unstimulated) or presence of anti-CD3, and plate-immobilized control Abs (rat IgG1/hamster IgG mixture), anti-4-1BB, or anti-CD28 mAb. Cultures were pulsed for the final 8 h of a 48-h culture period. Results are expressed in cpm. Error bars indicate SD. Note that the graph at 48 h has been truncated to preserve the same scale as graphs at other time points.

The results of a representative experiment appear in Figure 6. Consistent with other reports (17, 18), previously activated T cells died readily when reactivated in the absence of costimulation. As seen in Figure 6, although 51% of the cells are alive by 16 h, this percentage drastically drops to 24.8% by 24 h. Moreover, there is no increase in total cell number throughout the test period. Under the microscope, cells reactivated in the absence of costimulation show the characteristic morphologic changes associated with apoptosis (39–41): membrane blebbing with generation of apoptotic bodies, and a concomitant decrease in cell size (data not shown). In contrast, in the presence of either anti-4-1BB or anti-CD28 mAb, there is a significant increase in the total cell number between 16 and 84 h (from 2.46×10^5 to 7.55×10^5 cells for cultures reactivated in the presence of anti-4-1BB mAb; from 2.48×10^5 to 8.1×10^5 for reactivation with anti-CD28 mAb). Hence, the observed increases in the total cell population are consistent with the proliferative responses determined through [^{3}H]thymidine incorporation (Figs. 3–5). In addition, the percentage of live cells after 48 h of reactivation is nearly 70% for either 4-1BB or CD28 signaling, but only 16.9% for cells reactivated in the presence of control mAb. Importantly, after 48 h of reactivation, there is little, if any, apoptosis observed upon microscopic inspection (data not shown).

Anti-4-1BB costimulation prevents DNA fragmentation

In agreement with previous reports (17, 18), trypan blue staining/counting experiments revealed that splenic T cells readily died when reactivated. Furthermore, direct microscopic observations suggested that these cells were dying by apoptosis (data not shown). To confirm that cells were indeed dying by apoptosis, we conducted the following experiments.

One of the hallmarks of apoptotic cell death in most cell types (including lymphocytes) is the fragmentation of nuclear DNA (39–41). When genomic DNA isolated from cells undergoing apoptosis is electrophoresed on agarose gels, a characteristic 200-bp ladder is observed (39–41). However, besides being poorly quantitative, this method has the additional disadvantage that it does not provide information at the single cell level. An alternative quantitative approach is the terminal transferase dUTP nick-end labeling method (TUNEL). Following this procedure, DNA fragmentation was determined in reactivated splenic T cells. The results of two independent and representative experiments are presented in the histograms that appear in Figure 6. Splenic T cells were reactivated after 36 h (Fig. 7*A*) and 72 h of primary activation (Fig. 7*B*). As early as 36 h, DNA fragmentation was detected in nearly 30% of the surviving cell population. In contrast, cells that were costimulated with anti-4-1BB or anti-CD28 mAb presented little fragmentation (5.5 and 4.7%, respectively). Furthermore, after 72 h of primary culture, 97.5% of the cells exhibited DNA fragmentation when reactivated in the absence of costimulation, whereas only minimal fragmentation was detected in the presence of either anti-4-1BB or anti-CD28 mAb (1.1 and 6.5%, respectively).

Interestingly, the dot plots that appear to the left of each DNA-fragmentation histogram strongly support the previously presented cell-viability findings (Fig. 6). Dots within the upper region (R1)

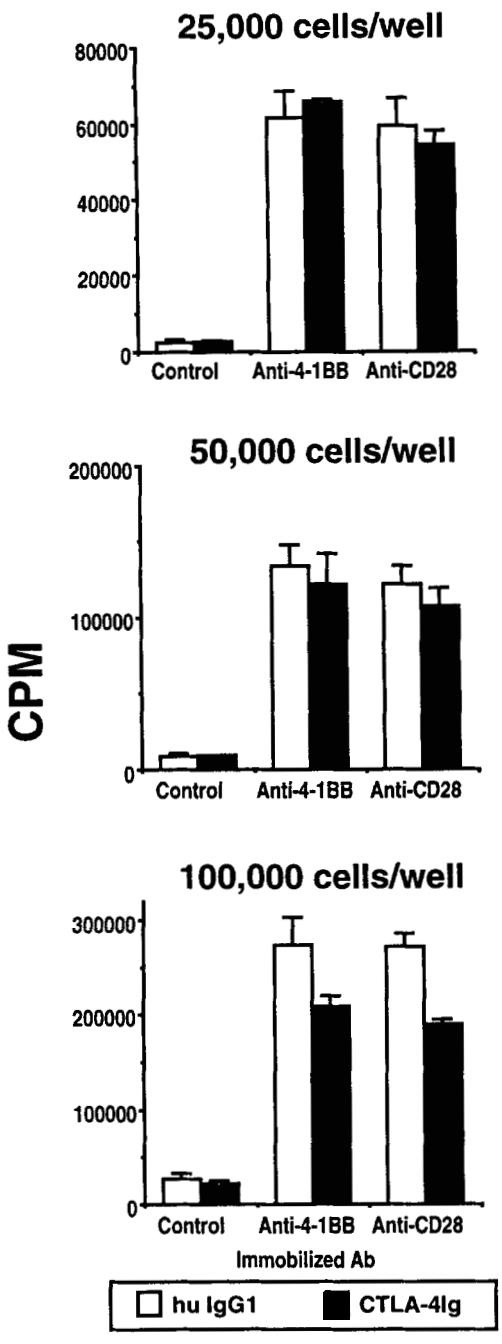

FIGURE 5. Anti-4-1BB mAb costimulates splenic T cells in the absence of B7 costimulatory signals. After 72 h of primary activation, the indicated number of splenic T cells was reactivated as indicated in Figure 3, but in the additional presence of CTLA-4Ig or its control human IgG1 at a final concentration of 10 μg/ml. Cultures were pulsed for the final 8 h of a 48-h culture period. Results are expressed as cpm. Error bars indicate SD.

are assumed to represent live cells, whereas dots within the lower region (R2) are assumed to correspond to dead cells and debris (36). These assumptions are based on forward (size) vs side scatter (granularity) considerations (36). Dot-plot analysis revealed that the percentage of live cells in reactivation cultures of T cells preactivated for 36 h was lower in the absence of costimulation: 40% vs 82.1% and 73.9% in the presence of anti-4-1BB, or anti-CD28 mAb, respectively. Likewise, in reactivation cultures of T cells preactivated for 72 h, the percentage of live cells in the absence of costimulation was 10.2%, but close to 70% in the presence of either costimulatory signal.

Discussion

The purpose of the current study was to evaluate the potential costimulatory function induced by 4-1BB engagement in the reactivation of previously stimulated T cells. To accomplish this goal, we established an in vitro procedure in which accessory signals were provided with immobilized mAbs directed against the T cell Ag 4-1BB or the prototypic costimulatory molecule CD28. Our findings indicate that cross-linking either receptor generates positive costimulatory signals that enhance anti-CD3-triggered proliferative responses and cytokine secretion in preactivated splenic T cells. Additionally, and as previously reported for CD28 (18, 19, 22), 4-1BB-mediated costimulation interferes with the apoptotic cell death triggered by recross-linking the TCR in the absence of other costimulatory signals.

The saturation kinetics of anti-4-1BB mAb treatment presented in Figure 3 suggests that the generated signals are specific for the Ab. Moreover, the time dependency of anti-4-1BB-mediated costimulation presented in Figure 4 partially supports this contention. Indeed, not taking into consideration the 48-h time point, maximal costimulation is observed following a 72-h preactivation: an activation time period that approximately corresponds with maximal 4-1BB expression (Fig. 2, 64 h). Costimulation decreases following a 96-h preactivation, and is lowest following a 120-h preactivation: a time point at which 4-1BB expression, although detectable, has dropped considerably (Fig. 2, 110 h). Although the absolute level of anti-4-1BB costimulation is greatest at the 48-h time point, it is noteworthy to indicate that additional factors may render this absolute value unsuitable for comparisons with the rest of the preactivation time points. Indeed, the dramatic drop in overall levels of reactivation observed between the 48- and 72-h preactivations suggests that there is something remarkably different about reactivation of cells preactivated for only 48 h. One potential factor is the presence of contaminating non-T cells (B cells, macrophages) at the 48-h time point, which are absent at the later time points (Fig. 1), and which may be providing a variety of potentially costimulatory or accessory signals that lead to the enhanced proliferative responses observed. Consistent with this interpretation are both the much higher background proliferation observed at 48 h, and the fact that both anti-4-1BB and anti-CD28 responses are in the same order of magnitude at 48 h and experience a similar drop by 72 h (Fig. 4). Another potential factor is the activation-induced expression of negative regulatory molecules by 72 h (and later) that may bring about reduced T cell proliferative responses. One candidate interaction to account for this regulatory function is that of activation-induced CTLA-4 with B7.1/B7.2 ligands (19, 42). However, as reported by Walunas and collaborators, maximal expression of CTLA-4 following primary activation is observed at 48 h, decreases significantly by 72 h, and reaches nearly background levels by 96 h (42): a pattern of expression that is not consistent with the drop in proliferation reported in this work. Furthermore, addition of the blocking reagent CTLA-4Ig to reactivation cultures does not enhance proliferation following preactivation for 64 h (Fig. 5): a finding that suggests that the drop in the response is not due to this molecular interaction. Nevertheless, it cannot be ruled out that other unidentified activation-induced negative regulators play a role in this phenomenon.

As indicated above, both the saturation kinetics and the partial time dependency of anti-4-1BB costimulation suggest that signals are specific for the Ab. However, one could rightly argue that the reported effect could be due to Ab cross-reactivity with other TCR.

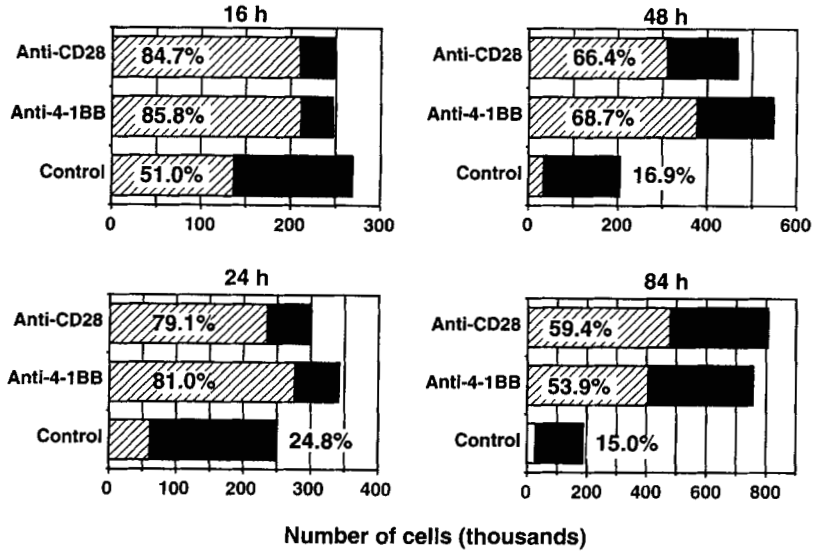

FIGURE 6. AICD in the presence of costimulatory signals. Previously activated splenic T cells were reactivated in the presence of plate-immobilized anti-CD28, anti-4-1BB, or control (rat IgG1) mAb. Cells were harvested after the indicated time periods, and cell viability and number were determined by microscopic observation after trypan blue staining. Cross-hatched bars represent live cells, while filled bars represent dead cells. The number within or next to each composite bar indicates the percentage of live cells.

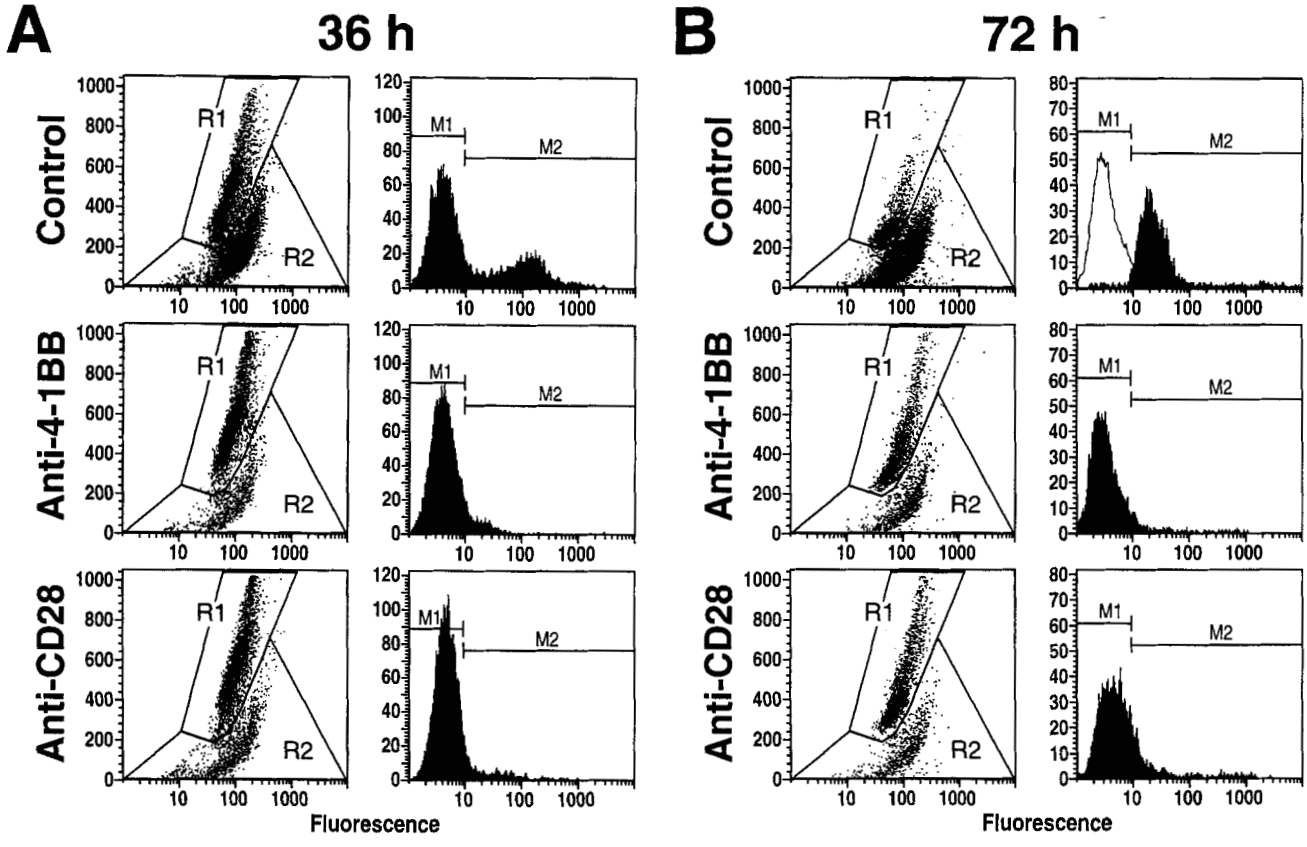

FIGURE 7. DNA fragmentation in splenic T cells reactivated in the presence of costimulatory signals. After 36 h (*A*) or 72 h (*B*) of primary activation, splenic T cells were isolated and reactivated in the presence of plate-immobilized control Abs (rat IgG1/hamster IgG mixture; *top panels*), anti-4-1BB (*middle panels*), or anti-CD28 (*bottom panels*) mAbs. After an additional 36-h culture, cells were harvested and stained for the presence of DNA strand breaks. Figures on the *left side* within each panel are dot plots of forward vs side scatter parameters. Figures to the *right* are histograms in which cells within the gate labeled M2 were considered positive. The empty histogram (*B, top panel*) corresponds to cells stained in the absence of TdT (*B*).

However, as already indicated in this work, 4-1BB-deficient mice failed to respond to signals provided by anti-4-1BB mAb (unpublished results). In addition, Abs generated against human 4-1BB are also capable of generating strong costimulatory signals in the activation of naive T cells (33) and in the reactivation of previously activated T cells isolated from human peripheral blood (Young-J. Kim, manuscript in preparation). Taken together, we conclude that the signals are very likely to be specific for 4-1BB.

Our current understanding of apoptosis is still in its infancy. In spite of tremendous research efforts and recent publications in the

field, there is still no unifying model that explains how apoptosis is induced or how it actually proceeds. These issues are complicated by the wide variety of model systems utilized by different research groups that include, for example, different inducing agents, cells, and culture conditions. Relating to T cell biology and the immune system, immunologists are presented with an inevitable paradox: activation signals induce proliferative responses under certain conditions and apoptotic cell death under others. In an attempt to explain this dichotomy, Green and Scott recently proposed two alternative causal mechanisms: cells undergo apoptosis because they receive irreversible death signals; or cells undergo apoptosis following activation unless they receive survival signals (43).

The first proposition is supported by recent reports that suggest that the interaction of Fas with its ligand is responsible for the induction of AICD in T cell lines, clones, hybridomas, and activated T cells (23, 44–46). Moreover, Russell and collaborators showed that activated T cells isolated from Fas- or FasL-defective mice (*lpr/lpr* or *gld/gld*, respectively) failed to undergo AICD in vitro (47, 48). However, these findings have been challenged by others who reported that TCR cross-linking on activated T cells of *lpr/lpr* mice does undergo apoptosis (14, 16). These seemingly contradictory results may find reconciliation in the findings of Zheng and collaborators, who recently indicated that AICD observed in *lpr/lpr* mice is mediated by TNF (16). In agreement with these findings, Sarin and co-workers recently reported that rTNF or lymphotoxin shares with FasL the ability to trigger AICD in in vitro activated T cells in both murine and human systems (15). Why one death pathway is favored over the other under different experimental conditions remains unknown. Relating to our model system, it appears that death signals arising from the interaction of Fas with its ligand are not responsible for the observed AICD. Indeed, FasFc, a functional antagonist of Fas that has been reported to efficiently block Fas-dependent AICD in other model systems (44–46, 49), has no effect in the apoptotic cell death induced following TCR cross-linking in our model system (unpublished results).

In contrast, it appears that 4-1BB-mediated costimulation prevents AICD, at least in part through promoting the secretion of IL-2 that may act as a potential survival signal in our model system. As already mentioned, anti-IL-2-neutralizing Abs had a very pronounced inhibitory effect on proliferation and cell survival (data not shown), induced by 4-1BB cross-linking. In agreement with this observation, IL-2 has been shown by others to prevent AICD in human medullary thymocytes (50, 51), AICD in mature activated T cells (19, 50), AICD of Th2-polarized effector cells (52, 53), and apoptosis triggered by signals other than TCR cross-linking (25, 26, 54). Interestingly, IL-2 has been shown to prevent AICD by generating survival signals (50) that counteract the otherwise lethal effect of IFN-γ secreted by activated T cells or Th1 clones following TCR re-engagement (50, 55, 56).

Although we presented evidence suggesting that IL-2 may be responsible for providing potential survival signals, additional mechanisms may also play a role. Indeed, recent independent reports have suggested that CD28 costimulation prevents apoptosis through enhancing the expression of the survival gene *bcl-*x_L (18, 25, 26). Interestingly, two reports indicated that both IL-2 (as an extrinsic survival factor) and *bcl-*x_L (as an intrinsic survival factor) participate in this protection (25, 26). However, the correlation between CD28 costimulation and *bcl-*x_L expression remains unclear. As presented by Radvanyi and co-workers, *bcl-*x_L expression is also induced following TCR cross-linking in the absence of costimulation, although it is reduced significantly at the onset of apoptosis (18). Additionally, Van Parijs and co-workers, using cells isolated from TCR transgenic mice, have reported recently that AICD of previously activated T cells cannot be prevented by *bcl-*x_L (23). Others in our laboratory are currently investigating these and other mechanisms that may help clarify how 4-1BB-mediated costimulation prevents AICD in our model system.

The role of alternative costimulatory signals (other than CD28) in the activation of naive T cells has been well documented for various Ig and integrin superfamily members (57–65), for heat stable Ag (66, 67), and for various TNFR superfamily members (67–74), including 4-1BB (30, 33, 34). However, there are no reports that implicate any of these interactions as positive costimulators in the reactivation of previously stimulated T cells. ICAM-1 and VCAM-1 are two Ig superfamily members that have been reported to provide potent costimulatory signals to resting T cells (58, 59, 61, 62). However, Damle and co-workers reported that in contrast to the positive costimulatory effect of B7, costimulation of Ag-specific T cell clones with either ICAM-1 or VCAM-1 enhanced AICD triggered by TCR cross-linking in a proportion of the stimulated population (75). Similarly, Gribben and collaborators reported that cross-linking of CTLA-4 with anti-CTLA-4 mAbs induced apoptosis in previously activated T cells isolated form human peripheral blood (19). In contrast, CD8 (Fig. 3*A*) (17), CD4 (17), or class I MHC (17) cross-linking fails to induce proliferative responses or to rescue preactivated T cells from AICD. Interestingly, CD4, CD8, and LFA-1 cross-linking was reported to prevent glucocorticoid-induced apoptosis of immature thymocytes (76).

In summary, CD28-mediated (18, 19, 25, 27) and 4-1BB-mediated costimulation (this study) have been shown capable of inducing proliferation and cell survival during the reactivation of previously stimulated T cells. Future research should provide answers relating to the various other putative costimulatory molecules identified to date. At present, the physiologic relevance of AICD in immune regulation and homeostasis remains unclear. However, an understanding of how different molecular interactions affect this process should aid in elucidating its significance and mode of action. Furthermore, the knowledge acquired relating to the interactions that induce or prevent AICD may have direct applications in the elaboration of immunotherapies for the control of tumor growth, autoimmunity, and other related immunologic disorders. One excellent example of the potential applicability of knowledge in this area comes from a recent report by Levine and co-workers (77). These researchers found that HIV-1-infected $CD4^+$ T cells could be grown and expanded ex vivo for periods of at least 50 days, if cultured with anti-CD3 in the presence of CD28 cross-linking; moreover, cells grown under these conditions gradually cleared HIV-1 infection. In addition, CD28-mediated costimulation in vitro rendered $CD4^+$ T cells resistant to subsequent HIV-1 infection (77). Although the antiviral mechanisms triggered by this procedure remain unknown, their identification holds great promise for combined immune reconstitution and HIV-1 clearance in affected individuals.

References

1. Janeway, C. A., Jr., and K. Bottomly. 1994. Signals and signs for lymphocyte responses. *Cell 76:275.*
2. Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature 346:425.*
3. Schwartz, R. H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science 248:1349.*
4. Schwartz, R. H. 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell 71: 1065.*
5. Allison, J. P. 1994. CD28-B7 interactions in T-cell activation. *Curr. Opin. Immunol. 6:414.*

6. Bluestone, J. A. 1995. New perspectives of CD28-B7-mediated T cell costimulation. *Immunity 2:555.*
7. June, C. H., J. A. Bluestone, L. M. Nadler, and C. B. Thompson. 1994. The B7 and CD28 receptor families. *Immunol. Today 15:321.*
8. Thompson, C. B. 1995. Distinct roles for the costimulatory ligands B7-1 and B7-2 in T helper cell differentiation? *Cell 81:979.*
9. Jenkins, M. K. 1994. The ups and downs of T cell costimulation. *Immunity 1:443.*
10. Linsley, P. S. 1995. Distinct roles for CD28 and cytotoxic T lymphocyte-associated molecule-4 receptors during T cell activation? *J. Exp. Med. 182:289.*
11. Linsley, P. S., and J. A. Ledbetter. 1993. The role of the CD28 receptor during T cell responses to antigen. *Annu. Rev. Immunol. 11:191.*
12. June, C. H., J. A. Ledbetter, P. S. Linsley, and C. B. Thompson. 1990. Role of the CD28 receptor in T-cell activation. *Immunol. Today 11:211.*
13. Sagerström, C. G., E. M. Kerr, J. P. Allison, and M. M. Davis. 1993. Activation and differentiation requirements of primary T cells in vitro. *Proc. Natl. Acad. Sci. USA 90:8987.*
14. Mogil, R. J., L. Radvanyi, R. Gonzalez-Quintial, R. Miller, G. Mills, and D. R. Green. 1995. Fas (CD95) participates in peripheral T cell deletion and associated apoptosis in vivo. *Int. Immunol. 7:1451.*
15. Sarin, A., M. Conan-Cibotti, and P. A. Henkart. 1995. Cytotoxic effect of TNF and lymphotoxin on T lymphoblasts. *J. Immunol. 155:3716.*
16. Zheng, L., G. Fisher, R. E. Miller, J. Peschon, D. H. Lynch, and M. J. Lenardo. 1995. Induction of apoptosis in mature T cells by tumour necrosis factor. *Nature 377:348.*
17. Radvanyi, L. G., G. B. Mills, and R. G. Miller. 1993. Religation of the T cell receptor after primary activation of mature T cells inhibits proliferation and induces apoptotic cell death. *J. Immunol. 150:5704.*
18. Radvanyi, L. G., Y. Shi, H. Vaziri, A. Sharma, R. Dhala, G. B. Mills, and R. G. Miller. 1996. CD28 costimulation inhibits TCR-induced apoptosis during a primary T cell response. *J. Immunol. 156:1788.*
19. Gribben, J. G., G. J. Freeman, V. A. Boussiotis, P. Rennert, C. L. Jellis, M. Barber, V. A. Restivo, Jr., X. Ke, G. S. Gray, and L. M. Nadler. 1995. CTLA4 mediates antigen-specific apoptosis of human T cells. *Proc. Natl. Acad. Sci. USA 92:811.*
20. Wesselborg, S., O. Janssen, and D. Kabelitz. 1993. Induction of activation-driven death (apoptosis) in activated but not resting peripheral blood T cells. *J. Immunol. 150:4338.*
21. Shi, Y., L. G. Radvanyi, A. Sharma, P. Shaw, D. R. Green, R. G. Miller, and G. B. Mills. 1995. CD28-mediated signaling in vivo prevents activation-induced apoptosis in the thymus and alters peripheral lymphocyte homeostasis. *J. Immunol. 155:1829.*
22. Groux, H., G. Torpier, D. Monte, Y. Mouton, A. Capron, and J. C. Ameisen. 1992. Activation-induced death by apoptosis in $CD4^+$ T cells from human immunodeficiency virus-infected asymptomatic individuals. *J. Exp. Med. 175:331.*
23. Van Parijs, L., A. Ibraghimov, and A. K. Abbas. 1996. The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. *Immunity 4:321.*
24. Noel, P. J., L. H. Boise, J. M. Green, and C. B. Thompson. 1996. CD28 costimulation prevents cell death during primary T cell activation. *J. Immunol. 157:636.*
25. Boise, L. H., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, and T. Lindsten. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity 3:87.*
26. Mueller, D. L., S. Seiffert, W. Fang, and T. W. Behrens. 1996. Differential regulation of *bcl*-2 and *bcl*-x by CD3, CD28, and the IL-2 receptor in cloned $CD4^+$ helper T cells: a model for the long-term survival of memory cells. *J. Immunol. 156:1764.*
27. King, C. L., R. J. Stupi, N. Craighead, C. H. June, and G. Thyphronitis. 1995. CD28 activation promotes Th2 subset differentiation by human $CD4^+$ cells. *Eur. J. Immunol. 25:587.*
28. Kwon, B. S., G. S. Kim, M. B. Prystowsky, D. W. Lancki, D. E. Sabath, and J. L. Pan. 1987. Isolation and initial characterization of multiple species of T-lymphocyte subset cDNA clones. *Proc. Natl. Acad. Sci. USA 84:2896.*
29. Pollok, K. E., S. H. Kim, and B. S. Kwon. 1995. Regulation of 4-1BB expression by cell-cell interactions and the cytokines, interleukin-2 and interleukin-4. *Eur. J. Immunol. 25:488.*
30. Pollok, K. E., Y. J. Kim, Z. Zhou, J. Hurtado, K. K. Kim, R. T. Pickard, and B. S. Kwon. 1993. Inducible T cell antigen 4-1BB: analysis of expression and function. *J. Immunol. 150:771.*
31. Hurtado, J. C., S. H. Kim, K. E. Pollok, Z. H. Lee, and B. S. Kwon. 1995. Potential role of 4-1BB in T cell activation: comparison with the costimulatory molecule CD28. *J. Immunol. 155:3360.*
32. DeBenedette, M. A., N. R. Chu, K. E. Pollok, J. Hurtado, W. F. Wade, B. S. Kwon, and T. H. Watts. 1995. Role of 4-1BB ligand in costimulation of T lymphocyte growth and its up-regulation on M12 B lymphomas by cAMP. *J. Exp. Med. 181:985.*
33. Alderson, M. R., C. A. Smith, T. W. Tough, T. Davis-Smith, R. J. Armitage, B. Falk, E. Baker, G. R. Sutherland, W. S. Din, and R. G. Goodwin. 1994. Molecular and biological characterization of human 4-1BB and its ligand. *Eur. J. Immunol. 24:2219.*
34. Goodwin, R. G., W. S. Din, T. Davis-Smith, D. M. Anderson, S. D. Gimpel, T. A. Sato, C. I. Brannan, N. G. Copeland, N. A. Jenkins, T. Farrah, R. J. Armitage, W. C. Fanslow, and C. A. Smith. 1993. Molecular cloning of a ligand for the inducible T cell gene 4-1BB: a member of an emerging family of cytokines with homology to tumor necrosis factor. *Eur. J. Immunol. 23:2631.*
35. Strober, W. 1992. Trypan blue exclusion test of cell viability. In *Current Protocols in Immunology.* J. E. Coligan, A. M. Kruisbeek, D. H. Margulis, E. M. Shevach, and W. Strober, eds. John Wiley & Sons, New York, pp. A.3.3-A.3.4.
36. Otten, G., and W. M. Yokohama. 1992. Immunofluorescence and cell sorting. In *Current Protocols in Immunology.* J. E. Coligan, A. M. Kruisbeek, D. H. Margulis, E. M. Shevach, and W. Strober, eds. John Wiley & Sons, New York, pp. 5.4.1–5.4.9.
37. Boehme, S. A., L. Zheng, and M. J. Lenardo. 1995. Analysis of the CD4 coreceptor and activation-induced costimulatory molecules in antigen-mediated mature T lymphocyte death. *J. Immunol. 155:1703.*
38. Azuma, M., D. Ito, H. Yagita, K. Okumura, J. H. Phillips, L. L. Lanier, and C. Somoza. 1993. B70 is a second ligand for CTLA-4 and CD28. *Nature 366:76.*
39. Cohen, J. J. 1993. Apoptosis. *Immunol. Today 14:126.*
40. Schwartz, L. M., and B. A. Osborne. 1993. Programmed cell death, apoptosis and killer genes. *Immunol. Today 14:582.*
41. Shi, Y. F., M. G. Szalay, L. Paskar, B. M. Sahai, M. Boyer, B. Singh, and D. R. Green. 1990. Activation-induced cell death in T cell hybridomas is due to apoptosis: morphologic aspects and DNA fragmentation. *J. Immunol. 144:3326.*
42. Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity 1:405.*
43. Green, D. R., and D. W. Scott. 1994. Activation-induced apoptosis in lymphocytes. *Curr. Opin. Immunol. 6:476.*
44. Brunner, T., R. J. Mogil, D. LaFace, N. J. Yoo, A. Mahboubi, F. Echeverri, W. R. Force, D. H. Lynch, C. F. Ware, and D. R. Green. 1995. Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature 373:441.*
45. Dhein, J., H. Walczak, C. Baumler, K. M. Debatin, and P. H. Krammer. 1995. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature 373:438.*
46. Ju, S. T., D. J. Panka, H. Cui, R. Ettinger, M. el-Khatib, D. H. Sherr, B. Z. Stanger, and A. Marshak-Rothstein. 1995. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature 373:444.*
47. Russell, J. H., and R. Wang. 1993. Autoimmune *gld* mutation uncouples suicide and cytokine/proliferation pathways in activated, mature T cells. *Eur. J. Immunol. 23:2379.*
48. Russell, J. H., B. Rush, C. Weaver, and R. Wang. 1993. Mature T cells of autoimmune *lpr/lpr* mice have a defect in antigen-stimulated suicide. *Proc. Natl. Acad. Sci. USA 90:4409.*
49. Alderson, M. R., T. W. Tough, T. Davis-Smith, S. Braddy, B. Falk, K. A. Schooley, C. A. Smith, F. Ramsdell, and D. H. Lynch. 1995. Fas ligand mediates activation-induced cell death in human T lymphocytes. *J. Exp. Med. 181:71.*
50. Groux, H., D. Monte, B. Plouvier, A. Capron, and J. C. Ameisen. 1993. CD3-mediated apoptosis of human medullary thymocytes and activated peripheral T cells: respective roles of interleukin-1, interleukin-2, interferon-gamma and accessory cells. *Eur. J. Immunol. 23:1623.*
51. Nieto, M. A., A. Gonzalez, A. Lopez-Rivas, F. Diaz-Espada, and F. Gambon. 1990. IL-2 protects against anti-CD3-induced cell death in human medullary thymocytes. *J. Immunol. 145:1364.*
52. Zhang, X., L. Giangreco, H. E. Broome, C. M. Dargan, and S. L. Swain. 1995. Control of CD4 effector fate: transforming growth factor beta 1 and interleukin 2 synergize to prevent apoptosis and promote effector expansion. *J. Exp. Med. 182:699.*
53. Muralidhar, G., S. Koch, H. E. Broome, and S. L. Swain. 1996. TCR triggering of anergic CD4 T cells in murine AIDS induces apoptosis rather than cytokine synthesis and proliferation. *J. Immunol. 157:625.*
54. Nakayama, K., I. Negishi, K. Kuida, H. Sawa, and D. Y. Loh. 1994. Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. *Proc. Natl. Acad. Sci. USA 91:3700.*
55. Liu, Y., and C. A. Janeway, Jr. 1990. Interferon gamma plays a critical role in induced cell death of effector T cell: a possible third mechanism of self-tolerance. *J. Exp. Med. 172:1735.*
56. Liu, Y., and C. A. Janeway, Jr. 1991. Monoclonal antibodies against T cell receptor/CD3 complex induce cell death of Th1 clones in the absence of accessory cells. *Adv. Exp. Med. Biol. 292:105.*
57. Sperling, A. I., J. M. Green, R. L. Mosley, P. L. Smith, R. J. DiPaolo, J. R. Klein, and C. B. Thompson. 1995. CD43 is a murine T cell costimulatory receptor that functions independently of CD28. *J. Exp. Med. 182:139.*
58. Damle, N. K., K. Klussman, P. S. Linsley, A. Aruffo, and J. A. Ledbetter. 1992. Differential regulatory effects of intercellular adhesion molecule-1 on costimulation by the CD28 counter-receptor B7. *J. Immunol. 149:2541.*
59. Damle, N. K., K. Klussman, and A. Aruffo. 1992. Intercellular adhesion molecule-2, a second counter-receptor for CD11a/CD18 (leukocyte function-associated antigen-1), provides a costimulatory signal for T-cell receptor-initiated activation of human T cells. *J. Immunol. 148:665.*
60. Linsley, P. S., J. L. Greene, P. Tan, J. Bradshaw, J. A. Ledbetter, and C. Anasetti. 1992. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. *J. Exp. Med. 176:1595.*
61. Davis, L. S., N. Oppenheimer-Marks, J. L. Bednarczyk, B. W. McIntyre, and P. E. Lipsky. 1990. Fibronectin promotes proliferation of naive and memory T cells by signaling through both the VLA-4 and VLA-5 integrin molecules. *J. Immunol. 145:785.*
62. Damle, N. K., K. Klussman, G. Leytze, H. D. Ochs, A. Aruffo, and P. S. Linsley. 1993. Costimulation via vascular cell adhesion molecule-1 induces in T cells increased responsiveness to the CD28 counter-receptor B7. *Cell. Immunol. 148: 144.*
63. Sato, T., K. Tachibana, Y. Nojima, N. D'Avirro, and C. Morimoto. 1995. Role of the VLA-4 molecule in T cell costimulation: identification of the tyrosine phosphorylation pattern induced by the ligation of VLA-4. *J. Immunol. 155:2938.*

64. Wacholtz, M. C., S. S. Patel, and P. E. Lipsky. 1989. Leukocyte function-associated antigen 1 is an activation molecule for human T cells. *J. Exp. Med. 170: 431.*
65. Cocks, B. G., C. C. Chang, J. M. Carballido, H. Yssel, J. E. de Vries, and G. Aversa. 1995. A novel receptor involved in T-cell activation. *Nature 376:260.*
66. Liu, Y., B. Jones, W. Brady, C. A. Janeway, Jr., and P. S. Linsley. 1992. Costimulation of murine CD4 T cell growth: cooperation between B7 and heat-stable antigen. *Eur. J. Immunol. 22:2855.*
67. Liu, Y., B. Jones, A. Aruffo, K. M. Sullivan, P. S. Linsley, and C. A. Janeway, Jr. 1992. Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. *J. Exp. Med. 175:437.*
68. Alderson, M. R., R. J. Armitage, E. Maraskovsky, T. W. Tough, E. Roux, K. Schooley, F. Ramsdell, and D. H. Lynch. 1993. Fas transduces activation signals in normal human T lymphocytes. *J. Exp. Med. 178:2231.*
69. Goodwin, R. G., M. R. Alderson, C. A. Smith, R. J. Armitage, T. VandenBos, R. Jerzy, M. A. Schoenborn, T. Davis-Smith, K. Hennen, B. Falk, D. Cosman, E. Baker, G. R. Sutherland, K. H. Gralistein, T. Farrah, J. G. Giri, and M. P. Beckmann. 1993. Molecular and biological characterization of a ligand for CD27 defines a new family of cytokines with homology to tumor necrosis factor. *Cell 73:447.*
70. Fanslow, W. C., K. N. Clifford, M. Seaman, M. R. Alderson, M. K. Spriggs, R. J. Armitage, and F. Ramsdell. 1994. Recombinant CD40 ligand exerts potent biologic effects on T cells. *J. Immunol. 152:4262.*
71. Cayabyab, M., J. H. Phillips, and L. L. Lanier. 1994. CD40 preferentially costimulates activation of $CD4^+$ T lymphocytes. *J. Immunol. 152:1523.*
72. Hintzen, R. Q., S. M. Lens, K. Lammers, H. Kuiper, M. P. Beckmann, and R. A. van Lier. 1995. Engagement of CD27 with its ligand CD70 provides a second signal for T cell activation. *J. Immunol. 154:2612.*
73. Kobata, T., K. Agematsu, J. Kameoka, S. F. Schlossman, and C. Morimoto. 1994. CD27 is a signal-transducing molecule involved in $CD45RA^+$ naive T cell costimulation. *J. Immunol. 153:5422.*
74. Smith, C. A., H. J. Gruss, T. Davis, D. Anderson, T. Farrah, E. Baker, C. I. Brannan, N. G. Copeland, N. A. Jenkins, K. H. Gralistein, B. Gliniak, I. B. McAlister, W. Fanslow, M. Alderson, B. Falk, S. Gimpel, S. Gillis, W. S. Din, R. G. Goodwin, and R. J. Armitage. 1993. CD30 antigen, a marker for Hodgkin's lymphoma, is a receptor whose ligand defines an emerging family of cytokines with homology to TNF. *Cell 73:1349.*
75. Damle, N. K., K. Klussman, G. Leytze, A. Aruffo, P. S. Linsley, and J. A. Ledbetter. 1993. Costimulation with integrin ligands intercellular adhesion molecule-1 or vascular cell adhesion molecule-1 augments activation-induced death of antigen-specific $CD4^+$ T lymphocytes. *J. Immunol. 151:2368.*
76. Zhao, Y., and I. Makoto. 1996. Cross-linking of the TCR-CD3 complex with CD4, CD8 or LFA-1 induces an anti-apoptotic signal in thymocytes: the signal is canceled by FK506. *Int. Immunol. 7:1387.*
77. Levine, B. L., J. D. Mosca, J. L. Riley, R. G. Carroll, M. T. Vahey, L. L. Jagodzinski, K. F. Wagner, D. L. Mayers, D. S. Burke, O. S. Weislow, D. C. St. Louis, and C. H. June. 1996. Antiviral effect and ex vivo $CD4^+$ T cell proliferation in HIV-positive patients as a result of CD28 costimulation. *Science 272: 1939.*