In Vivo Triggering Through 4-1BB Enables Th-Independent Priming of CTL in the Presence of an Intact CD28 Costimulatory Pathway

Linda Diehl, Geertje J. D. van Mierlo, Annemieke T. den Boer, Ellen van der Voort, Marieke Fransen, Liesbeth van Bostelen, Paul Krimpenfort, Cornelis J. M. Melief, Robert Mittler, Rene E. M. Toes and Rienk Offringa

*J Immunol* 2002; 168:3755-3762; doi: 10.4049/jimmunol.168.8.3755
http://www.jimmunol.org/content/168/8/3755

**References**
This article cites 25 articles, 15 of which you can access for free at: http://www.jimmunol.org/content/168/8/3755.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
In Vivo Triggering Through 4-1BB Enables Th-Independent Priming of CTL in the Presence of an Intact CD28 Costimulatory Pathway


Triggering of 4-1BB, a member of the TNFR family, through in vivo administration of agonistic anti-4-1BB Ab delivers a powerful costimulatory signal to CTL. We found this signal to effectively replace the need for CD4+ T cell help in the cross-priming of tumor-specific CTL immunity. Furthermore, 4-1BB Ab can convert an otherwise tolerogenic peptide vaccine into a formulation capable of efficient CTL priming. Initial activation of naive CTL can occur in the absence of 4-1BB costimulation, but this signal permits increased survival of Ag-stimulated CTL. Because naive CTL do not express 4-1BB at their surface, susceptibility to 4-1BB triggering depends on prior up-regulation of this receptor. We show that this requires both stimulation of the TCR and CD28-dependent costimulation. Accordingly, blockade of the CD28-costimulatory pathway abrogates the capacity of agonistic anti-4-1BB Ab to trigger Th-independent CTL immunity. In conclusion, our data reveal that the 4-1BB-mediated survival signal is positioned downstream of Ag-specific TCR triggering and CD28-dependent costimulation of naive CTL. The powerful effects of 4-1BB triggering on the induction, amplification, and persistence of CTL responses provide a novel strategy for increasing the potency of vaccines against cancers. The Journal of Immunology, 2002, 168: 3755–3762.

A recently acquired key insight in the mechanism through which CD8+ CTL are activated is the license to kill concept (1–3). We have demonstrated this principle by analyzing the induction of CTL responses in C57BL/6 mice against completely allogeneic tumor cells expressing the human adenovirus type 5 (Ad5)4 early region 1 (E1). In this setting, priming of Ad5-specific CTL immunity critically depends on cross-presentation of Ags by host APCs. Th-depleted mice are not capable of CTL priming against Ad5 Ags. Our data implied that in vivo triggering of dendritic cells (DC) through their CD40 receptor, either by anti-CD40 Ab or their physiological counterpart CD40-ligand on activated Th cells, enables these APC to prime CTL (1). The cardinal effect of CD40 triggering is the maturation of DC, which endows these cells with the capacity to present Ags in the context of the costimulatory signals required for priming of naive CTL (4).

Of the different costimulatory pathways involved in T cell priming, the signal delivered by CD80 and CD86, which are expressed at high levels on mature DC, to the CD28 receptor on T cells has been investigated most extensively (reviewed in Refs. 5 and 6). Although this signal plays a pivotal role in T cell activation, a number of additional costimulatory pathways also contribute to this process. One of these involves the 4-1BB/4-1BBL receptor-ligand pair. 4-1BB is a member of the TNFR family and is expressed on activated CD8+ and CD4+ T cells (7, 8). Its natural ligand, 4-1BBL, is expressed on B cells, macrophages, and DC (9, 10). In vitro studies have shown that stimulation of T cells with agonistic anti-4-1BB Abs increased TCR-induced proliferation and cytokine production by both CD8+ and CD4+ T cells (7, 11). Accordingly, blocking of 4-1BB costimulation in vitro was shown to inhibit APC-mediated T cell stimulation (12, 13), whereas 4-1BBL-deficient mice showed a reduced capacity to raise CTL immunity against virus infections (14–16). Furthermore, administration of agonistic anti-4-1BB Ab in mice was shown to amplify the generation of CTL in a murine model for graft vs host disease (11), whereas injection of these Ab into tumor-bearing mice resulted in the rejection of poorly immunogenic tumors (17).

Most published data argue that 4-1BB triggering, rather than being strictly required for induction of T cell immunity, is part of a complex costimulatory signal at the APC/T cell interface involving multiple ligand/receptor pairs. Only the paper by Melero et al. (17) suggests that the presence of a costimulatory signal through 4-1BB may truly make the difference between failure and efficacy of antitumor immunity. This prompted us to investigate the effects of 4-1BB costimulation on the generation of antitumor CTL.
immunity in more detail. Our experiments, which study the induction of CTL immunity against Ad5E1 tumor cells, reveal that in vivo triggering of 4-1BB, similar to in vivo triggering of CD40, can indeed make the difference between CTL nonresponsiveness and priming. Both the CD40 signal to APC and the 4-1BB signal to CTL are equally efficient in enabling CTL priming in the absence of CD4+ T cell help. Interestingly, we found that action of this powerful 4-1BB signal requires the CD28-costimulatory pathway to be intact, indicating that 4-1BB signaling is positioned downstream of CD28 costimulation.

Materials and Methods

Cell lines and animals

All in vitro cultures and assays were performed in IMDM (Life Technologies, Paisley, U.K.) supplemented with 8% FCS, 5 × 10−5 M 2-ME, glutamine, and penicillin (culture medium). C57BL/6 (H-2b) mice were purchased from IFFA Credo (Paris, France).

Vaccinations

Peptides Ad5E1A234–243, Ad5E1B192–209, or the control peptide human papillomavirus 16 E741–57 were dissolved in DMSO and diluted in PBS. For peptide vaccination, mice were injected s.c. with 100 μg of peptide (20 μl) diluted in PBS mixed with 100 μl of IFA. For in vivo triggering through CD40 or 4-1BB, mice received 100 μg of the CD40-activating Ab FGK-45 i.v., or the activating anti-4-1BB Ab 3H3 (11) given i.p. in 200 μl PBS on days 0, 1, and 2 after immunization. In blocking experiments, mice received 200 μg of the control rat IgG2a 6E9 (directed against human gp39; Ref. 18), the anti-4-1BBL Ab 14B3, or CTLA4-Ig i.p. (19) on days 0, 2, 4, 6, and 8 after vaccination. The anti-4-1BBL Ab 14B3 was generated as described (11) by injecting rats with a soluble mouse 4-1BBL-CDS fusion protein (20).

In cross-presentation experiments, mice were injected s.c. with 8 × 106 Ad5E1 mouse embryo cells (MEC) derived from TAP knockout or B6 background. These cells are C57BL/6-derived (H-2b) MEC transfected with the Ad5E1 region containing both the E1A and E1B gene (21). Depletion of CD4+ T cells was conducted as follows: mice received 100 μg of the anti-CD4 Ab GK1.5 in 200 μl PBS i.p. on days −7, −5, and −3 before Ad5E1 MEC injection and depletion was sustained twice weekly.

Cytotoxicity assay

Splenocytes from immunized mice were cocultivated in 24-well plates at 5 × 106 cells/well with IFN-γ-treated Ad5E1 MEC at a ratio of 10:1. Following a 6-day coculture, viable lymphocytes were collected and tested for cytotoxicity on Europium-labeled target cells as described previously (21). The mean percentage specific lysis of triplicate wells was calculated as follows: percentage of specific lysis = [(cpm experimental release – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release)] × 100. The SD of triplicates was in all cases <5%. Target cells used in cytotoxicity assays are the murine T cell lymphomas RMA and EL-4, B6 MEC, and Ad5E1 MEC. All target cells are of B6 (H-2b) origin, but only the Ad5E1 MEC express the Ad5E1A and E1B CTL epitopes.

TCR-transgenic (Tg) T cells

Tg mice on B6 genetic background expressing the TCR derived from a CTL clone that recognizes the Ad5E1A peptide epitope in the context of H-2Dd have been described previously (22). Freshly isolated splenocytes from naive TCR-Tg mice (recombination-activating gene−/− background) were stained with FITC-labeled anti-CD8 Ab and PE-labeled D8/E1A-peptide tetramers (23). The fraction of tetramer-positive T cells averaged between 75 and 95% of the CD8+ population and 12 and 25% of the total splenocyte population. Based on these measurements, a total splenocyte suspension containing 5 × 106 tetramer-positive CD8+ T cells, was injected i.v. per mouse. Where indicated, CFSE-labeling was conducted as follows: splenocytes were isolated and erythrocytes were lysed with NH4Cl. Spleen cells were washed in cold PBS and resuspended at 10 × 106/ml and labeled with 0.5 μM CFSE at 37°C for 30 min. Then FCS was added to a final concentration of 5%. Cells were washed and injected in PBS.

Flow cytometry analysis

For flow cytometry analysis, FITC-, PE-, or APC-conjugated Abs against CD86, MHC class II, CD11c, CD8α, and IFN-γ (BD Pharmingen, San Diego, CA), anti-Thy1.2 and goat-anti-rat (Biotrend, Cologne, Germany), and anti-4-1BBL (14B3) were used.

For intracellular IFN-γ detection, spleen cells from immunized mice were cultured in vitro with Ad5E1-transformed tumor cells for 6 days. Viable cells were isolated, and 200,000–500,000 cells per well were stimulated for 24 h with 1 μg/ml E1B peptide or control peptide in 96-well plates. During the last 5 h of stimulation, 10 μg/ml brefeldin A was added. Next, cells were washed in PBA (PBS containing 0.5% BSA, 0.02% azide) and fixed in 4% paraformaldehyde for 10 min (all incubations were done on ice). Cells were washed in PBA and in PBA containing 0.1% saponin (PBA-sap) and permeabilized in PBA-sap supplemented with 10% FCS for 10 min. Staining was performed with Abs diluted in PBA-sap for 30 min, washed in PBA-sap, and fixed in 1% paraformaldehyde. Data acquisition and analysis was performed on a BD Biosciences FACSCalibur using CellQuest software (BD Biosciences, Mountain View, CA).

In vitro Ab stimulation

The 96-well plates were coated overnight with various concentrations of anti-CD3 Ab (145.2C11) with or without 5 μg/ml of anti-CD28 Ab (3N7) in PBS. Plates were washed twice with PBS and 200,000–500,000 naive spleen cells or purified CD8+ T cells were added to the wells. After 24 h incubation, the cells were analyzed by FACS.

CD8+ T cells were purified as follows: spleen cells were isolated and B cells were removed with goat anti-mouse IgG magnetic beads according to the manufacturer’s protocol (Polyscience, Warrington, PA). Then cells were incubated with anti-CD4 Ab (GK1.5, 20 μg/ml) and anti-B220 (6B2, 20 μg/ml) for 30 min on ice, washed three times, and incubated with 10× excess of goat-anti-rat IgG magnetic beads (Polyscience) for 30 min on ice and put on a magnet. Remaining nonlabeled cells were >80% CD8+, and were cultured on Ab-coated plates as described above.

Results

In vivo triggering through 4-1BB provides a license to kill naïve CTL

We have previously shown that in vivo CD40 triggering of APC through the administration of an agonistic anti-CD40 Ab can replace the need for CD4+ T helper activity in cross-priming of Ad5-specific CTL (1). This prompted us to investigate whether in the same experimental system signals downstream of CD40 triggering that orchestrate the cognate interaction between APC and CTL, could similarly permit Th-independent cross-priming of CTL. We injected Ad5E1-transformed MEC into syngeneic CD4-depleted mice, and therefore, are incapable of presenting the Ad5E1-encoded CTL epitopes in the context of their own MHC (21). Consequently, priming of Ad5E1-specific CTL in this setting relies on cross-presentation of tumor Ags by host APC. The induction of Ad5E1-specific CTL is CD4+ T cell-dependent, as mice depleted for CD4+ T cells fail to mount an E1B-specific CTL response (Fig. 1b). Systemic administration of agonistic anti-CD40 Ab in these CD4-depleted animals restored E1B-specific cross-priming (Fig. 1c; Ref. 1). Interestingly, systemic administration of an agonistic anti-4-1BB Ab resulted in equally efficient priming of E1B-specific CTL in the absence of CD4+ T cell help (Fig. 1d).

A different method of inducing Ag-specific CTL immunity against tumors is through vaccination with minimal peptide epitopes in IFA. Notably, vaccination with the epitopes derived from Ad5E1A and E1B induces CTL tolerance rather than priming, which is witnessed by the increased growth of tumors expressing these Ags in peptide-vaccinated mice as well as by functional deletion of infused peptide-specific CTL in such mice (22). We have previously described that this tolerogenic peptide vaccine can be converted into an immunogenic CTL-priming vaccine when administered in combination with agonistic anti-CD40 Ab (23). Therefore, we investigated whether a trigger through 4-1BB could permit CTL priming in this setting. We injected B6 mice with the E1A-peptide vaccine in combination with anti-4-1BB Ab, anti-CD40 Ab, or control Ab. Indeed, not only coinjection of anti-
CD40, but also coinjection of anti-4-1BB converted the tolerogenic peptide vaccine into an immunogenic formulation (Fig. 1, e–g). Similar results were obtained when E1A-specific CTL immunity was monitored by staining of splenocytes with H-2D^d/E1A peptide tetramers (not shown). Thus, in vivo triggering of 4-1BB can prevent the induction of peptide-specific tolerance, and instead result in the priming of a potent E1A-specific CTL response. Taken together, these data demonstrate that the 4-1BB signal efficiently provides CTL with a license to kill, and as such can replace the need for CD40 triggering through Th cells or administration of agonistic anti-CD40 Ab.

Anti-4-1BB Ab does not induce DC activation

Current knowledge of the expression patterns of CD40 and 4-1BB would argue that the signal provided by in vivo administration of anti-CD40 Ab is delivered to the APC, whereas that of anti-4-1BB Ab is delivered directly to the CTL. To exclude the possibility that the anti-4-1BB Ab, instead of directly stimulating Ag-specific T cells, would mediate its effect on CTL priming (Fig. 1) in an indirect fashion by activation of the Ag-presenting DC, we investigated whether injection of the anti-4-1BB Ab activates DC in vivo. B6 mice were injected with anti-4-1BB, anti-CD40, or control Ab, and the CD86 (B7.2) expression on CD11c^+ DC isolated from the spleens of these mice was analyzed. As expected (23), in vivo triggering of CD40 resulted in an enhanced expression of CD86, indicating in vivo activation of the DC. In contrast, after in vivo 4-1BB triggering or administration of control Ab, the expression of CD86 on DC was not increased above background (Fig. 2a). The failure of the anti-4-1BB Ab to induce DC activation is in accordance with the fact that neither immature nor mature DC express detectable levels of 4-1BB, implicating that DC are not receptive for 4-1BB triggering (Fig. 2b). Therefore, anti-4-1BB Ab does not appear to affect the DC population, but rather mediates its stimulatory effect on CTL priming through direct stimulation of CD8^+ T cells.

**FIGURE 1.** In vivo triggering through 4-1BB restores priming of Ad5-specific CTL in the absence of T cell help. B6 mice, either nondepleted (a) or depleted for CD4^+ T cells (b–d) were vaccinated with 10^7 TAP knockout Ad5E1 MEC s.c. in combination with control Ab (b), anti-CD40 Ab (c), or anti-4-1BB Ab (d). In a separate experiment, B6 mice received a dose of 20 μg E1A peptide in IFA either in combination with control Ab (e), anti-CD40 Ab (f), or anti-4-1BB Ab (g). Splenocytes from immunized mice were restimulated in vitro for 6 days and subsequently tested for Ag-specific cytotoxicity on target cells (RMA) loaded with the E1B peptide (a–d) or the E1A peptide (e–g; [9]), the human papillomavirus 16 E7 peptide (□), or Ad5E1 MEC (○). The stimulatory effects of 4-1BB Ab on Ad5-specific CTL priming by tumor cells or the E1A peptide have each been observed in three separate experiments. Overall, we found that anti-CD40 and anti-4-1BB Ab were equally capable of enabling Ad5-specific CTL priming under these conditions.

**FIGURE 2.** Systemic administration of anti-4-1BB Ab does not induce DC activation in vivo. a, B6 mice were injected 2 times with 100 μg of control Ab (light gray histograms), anti-CD40 Ab (dark gray, left), or the anti-4-1BB Ab (dark gray, right). Three days later spleen cells were isolated and stained with anti-CD11c and anti-CD86. Depicted are the CD86 expression levels on CD11c^+ cells in the spleen. b, B6 bone marrow was isolated and cultured in vitro for 7 days in GM-CSF-containing medium. On day 7, cells were activated by addition of 10 μg/ml poly(I:C), or left untreated. Forty-eight hours later, cells were analyzed for 4-1BB expression (black line) or stained with a control Ab (filled light gray). Activation of poly(I:C)-treated bone marrow DC was confirmed by measuring CD86 expression (not shown).
Costimulation through 4-1BB results in increased survival of Ag-stimulated CTL

To investigate in which manner agonistic anti-4-1BB Ab permits Th-independent priming of Ad5-specific CTL, we exploited adoptively transferred T cell populations from Ad5E1A peptide-specific TCR Tg mice (22). In contrast to the endogenous E1A-specific CTL response repertoire, the TCR Tg T cells allowed us to determine the fate of E1A-specific CTL upon immunization with the E1A peptide. Tracking of these T cells in vivo was performed in two distinct ways. First, TCR Tg T cells were labeled with CFSE and transferred into normal B6 mice, after which these mice were vaccinated with the Ad5E1A-peptide in IFA with or without anti-4-1BB Ab treatment. Interestingly, the resulting data revealed that proliferation of the TCR-Tg T cells was equally triggered in mice that had received the E1A peptide either with or without anti-4-1BB Ab (Fig. 3a). Therefore, anti-4-1BB Ab do not enable CTL priming by increasing the initial activation or proliferative capacity of Ag-specific T cells.

In a second approach, Ad5E1A-specific TCR Tg T cells from B6 Thy1.2 origin were transferred into B6 Thy1.1 congenic mice. These mice were vaccinated with the Ad5E1A-peptide in IFA with or without anti-4-1BB Ab treatment. Fig. 3b shows that 11 days after vaccination, massive expansion of CD8+ T cells from TCR-Tg origin was observed in mice vaccinated with E1A peptide in combination with anti-4-1BB Ab, but not in mice that received either E1A-peptide or anti-4-1BB Ab alone. Therefore, accumulation of E1A-specific CTL required the presence of the 4-1BB-costimulatory signal. In the absence of this signal, E1A-specific TCR Tg T cells were ultimately deleted in a manner similar to that observed for the endogenous E1A-specific CTL repertoire (Fig. 1, c–g and Refs. 22 and 23). Furthermore, stimulation of CD8+ CTL immunity through 4-1BB was dependent on the presence of an Ag-specific trigger. Analysis of the kinetics of the response of the TCR-Tg T cells reveals that administration of the antigenic trigger in the presence of 4-1BB costimulation results in a rapid accumulation of E1A-specific T cells, which even peaks around day 6 after immunization, followed by a gradual decline in specific T cell numbers. In contrast, no accumulation of E1A-specific T cells is detected at any of the time points tested, indication that T cell deletion in the absence of the 4-1BB signal is a very rapid and efficient process (Fig. 3c). Taken together, these data demonstrate that 4-1BB triggering is not essential for initial CTL activation, but promotes the survival of Ag-stimulated CTL, thereby permitting expansion of these CTL.

Involvement of 4-1BBL/4-1BB interactions in CTL priming

Because in vivo triggering of 4-1BB allows for CTL priming in the absence of the CD40-dependent DC activation signal (provided by either Th cells or administration of agonistic anti-CD40 Ab; Fig. 1), this implies that Ag presentation by nonactivated DC in combination with an activating anti-4-1BB Ab suffices for efficient CTL priming. The need for coadministration of anti-4-1BB Ab also indicated that nonactivated DC were apparently not capable of stimulating CTL through 4-1BB. Therefore, we analyzed expression of the natural ligand for this receptor, 4-1BBL, on immature and mature DC. Nonactivated DC express low levels of 4-1BBL, whereas the expression of this molecule is strongly increased as a result of activation through the CD40 receptor. This induction of 4-1BBL was observed upon CD40 triggering of DC in vivo (Fig. 4) and in vitro (data not shown). This increase in 4-1BBL expression was paralleled by elevated levels of CD86 on the activated DC. This suggests that both costimulatory signals contribute to the

FIGURE 3. Costimulation through 4-1BB is Ag-dependent and results in increased survival of Ag-specific T cells. a, B6 mice were injected with 5 × 106 Ad5E1A-specific TCR Tg, CFSE-labeled CD8+ T cells. Three days later, these mice received a combination of the E1A peptide and Ab as indicated in the figure. Peptide vaccination involved a single s.c. 20 μg dose in IFA, whereas 100 μg doses of Ab were administered i.p. on 3 consecutive days following peptide vaccination. At day 3 after injection of the peptide, spleen cells were isolated and analyzed by FACS for the presence of CD8+ CFSE+ T cells. The histogram shows the CFSE intensity of CD8+ T cells. b, B6 Thy1.1 mice were injected with 5 × 106 E1A peptide-specific TCR Tg Thy1.2+ CD8+ T cells. Three days later, these mice received a combination of E1A peptide and Ab as indicated in the figure (doses and timing as above). Eleven days after injection of the peptide vaccine, blood samples were taken and analyzed by FACS for the presence of CD8+Thy1.2+ T cells. The histogram shows the CFSE intensity of CD8+ T cells transferred into B6 Thy1.1 mice and then vaccinated (b) at the indicated time points (days). Each bar represents the average of two mice.
capacity of mature DC, as opposed to immature DC, to induce CTL immunity.

We performed tumor cell immunization experiments in normal, CD4\(^{+}\) Th cell-proficient B6 mice, in which the Th-dependent DC maturation signal required for CTL cross-priming is intact. Blockade of the CD28 and 4-1BB signals was performed by administration of blocking anti-4-1BBL Ab or CTLA4-Ig respectively (Fig. 5). B6 mice were depleted for CD4\(^{+}\) T cells and immunized with Ad5E1 MEC in combination with administration of anti-4-1BBL Ab and/or CTLA4-Ig. Interestingly, in vivo blockade of anti-4-1BBL resulted in a marked reduction in the number of Ag-specific CTL induced by the tumor cell vaccine. Blockade of CD28 costimulation completely inhibited CTL induction and, therefore, additional 4-1BBL blocking in combination with CTLA4-Ig had no extra effect. It should be noted that the reduction of the amount of CTL induced in the presence of 4-1BBL blockade was less apparent when analyzed in cytolytic assays, whereas CD28 blockade resulted in complete absence of target cell lysis. These data indicate that 4-1BBL/4-1BB interactions play a role in the cross-priming of Ag-specific CTL, as blocking of the interaction between these molecules diminishes the numbers of tumor-specific CTL that are sustained. However, in the absence of the costimulatory signal through CD28, provided by properly activated DC, the signal provided by 4-1BBL is apparently not capable of enabling CTL priming and, as a result, induction of antitumor CTL immunity is completely abrogated.

The 4-1BB signal depends on CD28 costimulation

The in vivo blocking experiments suggested a dominance of CD28 costimulation over the 4-1BB signal. Therefore, we investigated whether in the absence of CD28 costimulation, administration of anti-4-1BB Ab would still provide CTL with a license to kill. B6 mice were depleted for CD4\(^{+}\) T cells and immunized with Ad5E1 MEC in combination with administration of anti-4-1BB Ab and/or CTLA4-Ig. Mice that received anti-4-1BB Ab mounted strong E1B-specific CTL responses, but mice that also received CTLA4-Ig failed to do so (Fig. 6). These data indicate that the positive effect of 4-1BB triggering on the induction of CTL immunity is dependent on the presence of an intact CD28 costimulatory signal. Furthermore, these data suggest that the basal levels of CD80/CD86 expressed by nonactivated DC provide sufficient costimulation through CD28 to allow additional costimulation through the 4-1BB pathway. In vitro studies have demonstrated...
that 4-1BB expression on naive T cells is absent and that TCR triggering, through cross-linking by anti-CD3 Ab, results in a rapid increase in 4-1BB surface expression (7). As our data argue that costimulation through CD28 is prerequisite for 4-1BB signaling, we investigated whether CD28 triggering contributes to the up-regulation of 4-1BB on naive T cells. Therefore, naive total spleen cells or purified CD8⁺ T cells were stimulated in vitro with plate-bound anti-CD3 Ab and analyzed for 4-1BB expression 24 h later (Fig. 7). It is clear that strong signals through the TCR (high concentrations of anti-CD3 Ab) are sufficient to induce 4-1BB expression within 24 h. However, when T cells are stimulated with lower anti-CD3 concentrations, their capacity to up-regulate 4-1BB is largely lost (Fig. 7). These lower anti-CD3 Ab concentrations provide a weaker TCR trigger that is more likely to resemble an in vivo signal of the kind provided by the tumor cell vaccine. Importantly, under these weak CD3-triggering conditions, costimulation through the CD28 receptor restored the capacity of T cells to express high levels of 4-1BB. These findings are in accordance with the fact that blockade of the CD28 pathway in vivo abrogates costimulation through 4-1BB (Fig. 6). Furthermore, they strengthen the notion that CD28-costimulation of Ag-stimulated T cells is an important signal for 4-1BB up-regulation on naive T cells, thereby making these cells susceptible for 4-1BB triggering.

**Discussion**

Our cumulative data show that costimulation through 4-1BB is a powerful signal for the induction of CTL immunity, and constitute the first direct demonstration that the 4-1BB signal can make the difference between CTL nonresponsiveness and priming. In vivo triggering of 4-1BB results both in the induction of Ag-specific CTL immunity by an otherwise tolerogenic peptide vaccine (Fig. 1, e–g), and in cross-priming of tumor-specific CTL in the absence of CD4⁺ T cell help (Fig. 1, a–d). Furthermore, in vivo blockade of 4-1BB/L4-1BB interactions markedly decreases the efficacy of Th-dependent cross-priming of tumor-specific CTL (Fig. 5). Importantly, in vivo costimulation through CD28 is necessary for effective signaling through 4-1BB to take place (Figs. 5–7), indicating that the 4-1BB signal lies downstream of CD28 costimulation.

The effect of the anti-4-1BB Ab on the immunogenicity of the Ad5E1A peptide vaccine is as striking as the effect we previously reported for the agonistic anti-CD40 Ab (Fig. 1; Ref. 23). In both cases, systemic coadministration of the Ab converted the tolerogenic vaccine into a formulation capable of efficient CTL priming. This indicates that costimulation through 4-1BB is a promising novel approach for increasing the potency of vaccines. Importantly, the effect of in vivo 4-1BB triggering is conceptually distinct from that of in vivo CD40 triggering. Systemic administration of anti-CD40 Ab in combination with the E1A peptide vaccine results in the in vivo activation of the peptide-loaded DC, thereby endowing these DC with the capacity to prime E1A-specific CTL (23). In a similar fashion, administration of anti-CD40 Ab was shown replacing the need for CD4⁺ T cell help in cross-priming of CTL by an Ad5 MEC tumor cell vaccine (1). We now show that the agonistic anti-4-1BB Ab allows for CTL priming in these very same settings (Fig. 1), but mediates its immunostimulatory action by directly triggering the CTL, rather than indirectly through activation of DC (Fig. 2). This is in agreement with previous studies which revealed that agonistic anti-4-1BB Ab can augment T cell responses in vitro, in particular of CD8⁺ T cells (7, 11). Thus, the signal through 4-1BB acts downstream of CD40 signaling, and administration of agonistic anti-4-1BB Ab replaces the need of CD40-mediated APC activation by either Th cells or by agonistic anti-CD40 Ab. In accordance with this notion, we found that activation of DC by inflammatory agents or through CD40 triggering results in increased 4-1BBL expression (Fig. 4), implicating that only properly activated DC can provide a strong costimulatory signal to the 4-1BB receptor on T cells.

Analysis of the mechanism by which 4-1BB triggering enables the induction of CTL immunity revealed that this signal enhances the survival of Ag-stimulated T cells (Fig. 3). It was previously shown that triggering through 4-1BB results in the increased survival of anti-CD3-stimulated T cells in vitro as well as of superantigen-stimulated T cells in vivo (7, 8). We now demonstrate that this feature of 4-1BB also applies to the induction of Ag-specific, MHC-restricted CTL responses in vivo. Notably, the initial proliferation of T cells upon Ag exposure also occurs in the absence of 4-1BB costimulation (Fig. 3a). This implies that 4-1BB plays a
role more downstream in the process of T cell activation. Indeed, our experiments, in which we compared the roles of the costimulatory signals through 4-1BB and CD28, are in support of this. In particular, agonistic anti-4-1BB Ab failed to permit cross-priming of antitumor CTL in CD4+ T cell-depleted animals when the costimulatory signal through CD28 was blocked by CTLA4-Ig administration (Fig. 6). Furthermore, we show that TCR triggering in combination with CD28 costimulation is much more efficient in inducing surface expression of 4-1BB on T cells (Fig. 7) than TCR triggering alone. Thus, stimulation of naive T cells through their constitutively expressed TCR and CD28-receptor primes these T cells and at the same time makes these cells receptive for a survival signal through 4-1BB. Provision of this signal, either by 4-1BBL expressed on activated DC (Fig. 4) or by agonistic anti-4-1BB Ab, results in survival, and thereby, expansion of the Ag-primed T cells (Figs. 1 and 3); whereas lack of this survival signal will limit the magnitude of these responses (Fig. 5, Refs. 14–16).

Whereas our data indicate that delivery of the survival signal through 4-1BB depends on CD28 costimulation (Figs. 6 and 7), others have reported that 4-1BB triggering of T cells can also take place independently of the CD28 pathway. However, these studies all examined the role of the 4-1BB signal in combination with very strong TCR triggering, such as high concentrations of plate-bound anti-CD3 Ab or coculture of T cells with allogeneic APC (10, 13, 14, 24). Both our results (Fig. 7) and the data of others (7) have shown that strong TCR triggering in the absence of a signal through CD28 suffices to induce 4-1BB expression on naive T cells, thereby enabling 4-1BB costimulation of T cells independent of the CD28 pathway. Thus, our data are not in conflict with previously published reports concerning the relation between the CD28 and 4-1BB costimulatory pathways in the presence of very strong TCR triggering. Notably, in our study we have also looked at this relation in settings where the TCR trigger is weaker. Although high concentrations of anti-CD3 Ab suffice to induce 4-1BB expression of naive T cells, lower concentrations of these Ab fail to efficiently up-regulate 4-1BB, unless costimulation through CD28 is provided (Fig. 7). Accordingly, Saoulli et al. (25) reported that 4-1BB costimulation of resting T cells was only found when combined with high amounts of anti-CD3 Ab, not when combined with lower concentrations of anti-CD3 Ab. Most importantly, we now demonstrate that the dependency of the 4-1BB signal on the CD28 pathway also extends to an in vivo setting where a more physiological signal to the TCR is provided by a tumor cell vaccine. Although in vivo administration of agonistic anti-4-1BB Ab permits cross-priming of CTL against this tumor cell vaccine in the absence of CD4+ T cell help, this immunostimulatory effect of anti-4-1BB Ab is abrogated if the CD28 pathway is blocked (Fig. 6). Likewise, the signal provided by 4-1BBL on properly activated APC in CD4+ Th-proficient mice fails to potentiate CTL priming if CD28 costimulation is blocked (Fig. 5). Therefore, we conclude that under physiological conditions, the delivery of the 4-1BB-mediated survival clearly depends on the CD28 pathway.

The fact that blockade of CD28 costimulation completely abolishes T cell cross-priming (Fig. 6) has interesting implications for our observation that agonistic anti-4-1BB Ab permits cross-priming in CD4+ T cell-depleted mice (Fig. 1). These data argue that even in the absence of CD4+ T cell help, the cross-presenting APC can provide costimulation through CD28, which in combination with the antigenic signal is sufficiently strong to sensitize the CTL for 4-1BB triggering. This is quite conceivable, as also immature DC express CD80 and CD86, albeit at much lower levels than their activated counterparts (Fig. 2; Ref. 4).

In summary, stimulation of CTL through 4-1BB is dependent on TCR triggering and CD28 costimulation. Engagement of the 4-1BBL/4-1BB pathway is important for the efficient induction of CTL immunity. Signaling through 4-1BB promotes survival of Ag-triggered CTL, thereby increasing the magnitude of the CTL response after vaccination with either tumor cells or minimal peptide epitopes. The effects of in vivo 4-1BB triggering are particularly dramatic in cases where insufficient CD4+ T cell help is available to properly activate APC. Our observations point at an important role for 4-1BB costimulation in the multistep activation of naive CTL by properly matured DC, and stress the potency of 4-1BB as a license to kill signal for improving the efficacy of vaccines.

Acknowledgments

We thank Dr. Marek Kubin (Immunex, Seattle, WA), Dr. Ton Schumacher (The Netherlands Cancer Institute, Amsterdam, The Netherlands), and Dr. Chris Larsen (Emory University, Atlanta, GA) for kindly providing purified recombinant murine CD40L trimer, D'/E1A tetramers, and CTLA4-Ig, respectively.

References