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References

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4-1BBL Cooperates with B7-1 and B7-2 in Converting a B Cell Lymphoma Cell Line into a Long-Lasting Antitumor Vaccine

Barbara-ann Guinn,* Mark A. DeBenedette,* Tania H. Watts,* and Neil L. Berinstein†‡§

A20 is a B cell lymphoma that constitutively expresses the costimulatory molecule B7-2 yet grows readily as a tumor in syngeneic BALB/c mice. We have compared the tumorigenicity of A20 variants expressing either B7-1 (A20/B7-1) or B7-2 (A20/B7-2) with an A20 variant expressing B7-1 and B7-2 with 4-1BBL (A20/4-1BBL), a costimulatory member of the TNF family. Mice injected with tumors expressing the vector backbone (A20/CMV) or B7-1 developed tumors within 25 days of s.c. injection. In contrast, mice injected with A20/4-1BBL were tumor free for the 150-day follow-up period, while 25% of mice injected with A20/B7-2 developed tumors. Tumorigenicity experiments using nude mice indicated the requirement for T cells for variant rejection. Almost all mice that resisted the initial tumor challenge were resistant to further challenge with the parental tumor. Splenocytes from these mice showed high CTL lytic activity against the parental tumor, A20, as well as the syngeneic BALB/c lymphoma K46J, but showed background levels of lytic activity against the congenic SCID thymoma line ST-D2 or the allogeneic EL4 thymoma. In vitro blocking experiments with anti-B7-1 plus anti-B7-2 and/or soluble 4-1BB receptor showed B7-1, B7-2, and 4-1BBL all contributed to the CTL activity. Thus, the data show that neither B7-1 or B7-2 alone can confer full immunogenicity to the A20 lymphoma but that the addition of 4-1BBL results in a tumor that is highly immunogenic and can confer long-lasting protection against challenge with parental tumor in vivo. The Journal of Immunology, 1999, 162: 5003–5010.

Costimulatory molecules on APCs have been shown to be the necessary second signal required to activate naïve T cells. The first signal comes from the binding of processed Ag presented on MHC molecules by the TCR/CD3 complex on T cells. The absence of the second signal has been shown to lead to T cell anergy (1, 2) and is thought to be one mechanism by which tumors evade the immune system (3, 4). The B7 family of costimulatory molecules consists of two members, B7-1 (CD80) and B7-2 (CD86) (5–8), which have been shown to be equally able to activate T cells. These molecules are generally expressed on APCs, including macrophages, dendritic cells, and activated B cells (5, 9, 10) and have more recently been shown to be expressed on T cells (11, 12). Most APCs require activation for the expression of B7-1 and B7-2, and B7-2 is induced more rapidly than B7-1 after various stimuli (13, 14). The receptors for the B7 molecules are CD28 and CTLA4. Positive signals that lead to T cell proliferation and cytokine secretion are mediated through CD28, while CTLA4 appears to transduce an “off” signal limiting the period of T cell activation and proliferation (15–18).

Another more recently described costimulatory receptor, 4-1BB (19, 20), has been shown to have a pattern of expression that follows the primary activation of T cells and is restricted to activated CD4+ and CD8+ T cells (20–22). Engagement of the 4-1BB receptor has been shown to relay strong costimulatory signals within activated T cells, which leads to their enhanced proliferation and cytokine secretion (23, 24). Such signaling prevents activation-induced cell death (25) following TCR cross-linking in the absence of other accessory signals. 4-1BBL (20) is a high-affinity ligand for 4-1BB, expressed on the surface of activated APCs (20, 21, 26, 27). It is a type II membrane protein that shows homology to members of the TNF receptor family (20, 28, 29). T cells purified from CD28−/− mice have been shown to secrete cytokines and proliferate in response to lymphomas expressing 4-1BBL, a response that can be inhibited by the soluble 4-1BB receptor fusion protein (27). In the absence of a CD28 signal, the 4-1BBL:4-1BB interaction has been shown to have a role in the production of a Th2 response in mixed lymphocyte reactions (MLR)3 (30).

A number of investigators have transfected B7-1 or B7-2 into tumor cells and assessed the affect on tumor immunogenicity. Most of these studies have shown that elevating B7-1 levels confers an antitumor response in a number of tumor cell lines (4, 31–34). While most studies have indicated that B7-1 was more potent than B7-2 at conferring antitumor immunity (34–36), some studies have shown that B7-2 was more effective in other model systems (37). The variation in antitumor response conferred by the B7 molecules appears to lie in the immunogenicity of the tumor cell line used (32, 37). In the present report, we analyze the efficacy of the 4-1BBL molecule in eliciting an antitumor immune response against A20 B cell lymphomas. Recent investigations have indicated the potential for the 4-1BB:4-1BBL interaction to confer antitumor immunity (38, 39), and we now show that transfected 4-1BBL in combination with B7-1 and B7-2 is more effective than cells transfected with either the B7-1 and B7-2 molecules alone in conferring antitumor immunogenicity in a model system. We also demonstrate that A20 cells that express high levels of 4-1BBL...
together with B7-1 and B7-2 are able to mediate full, long-lasting tumor immunogenicity preventing tumor development and producing an in vivo resistance to further tumor challenge, which is mediated through CTL activity. Thus, a combinatorial approach involving several costimulatory molecules may offer a more effective strategy for antitumor vaccine regimens.

**Materials and Methods**

**Cell lines, transfection, and cloning**

The A20 (40) BALB/c B cell lymphoma line originally derived from a spontaneous reticulum cell neoplasm type B was obtained from the American Type Culture Collection (ATCC, Manassas, VA). All A20 cells and variants were maintained as described previously (41). The full-length 4-1BBL cDNA was isolated by RT-PCR from the K46J cell line and subcloned into the pRc/CMV vector (Invitrogen, San Diego, CA) as described elsewhere (42). Cells were electroporated with 100 μg of plasmid, cloned, and subcloned as described previously (41). The A20/4-1BBL clone 1 (Cl.1) was derived from an independent clone to the A20/4-1BBL clone 2 (Cl.2).

The X63 IL-2- and IL-4-secreting cells lines were a kind gift from Dr. Fritz Melchers (Basel Institute for Immunology, Basel, Switzerland) (43). The CTLL-2 IL-2-dependent line was obtained from ATCC, and the CT.4S IL-4-dependent line was a kind gift from Dr. William Paul (National Institute of Allergy and Infectious Disease, Bethesda, MD). The BALB/c B cell lymphoma line K46J (40) and the C57BL/6 lymphoma line EL4 were obtained from ATCC. The SCID thymoma line ST-D2 was derived from a mouse with a congenic BALB/c background and was obtained from Dr. Gillian Wu (Ontario Cancer Institute, University of Toronto, Toronto, Canada).

**Abs and reagents**

The hybridomas used in these studies were obtained from ATCC and used as described previously (30). T cell phenotype checks before use in MLR were performed using anti-TCR, anti-CD4, anti-CD8 (PharMingen, San Diego, CA), and anti-CD3 (Cedarlane Laboratories, Hornby, Ontario, Canada) Abs in addition to those described above. FITC-conjugated anti-mouse IgG (Sigma) and streptavidin-FITC (Molecular Probes, Eugene, OR) were used as secondary Abs, the latter in conjunction with n-biotin (Molecular Probes)-conjugated (44) primary Abs. Isotype controls of rat IgG2a and rat IgG2b were obtained from Cedarlane, and murine IgG2a was obtained from PharMingen and used at equivalent concentrations to test Abs in each experiment.

NIH3T3 cells secreting the fusion protein of the extracellular domain of 4-1BB linked to alkaline phosphatase (AP) were a kind gift from Dr. Byoung Kwon (Indiana University, Indianapolis, IN) (26). 4-1BB-AP was purified as described previously (27), and AP was used as an irrelevant binding control.

**Cell counts and phenotyping**

Cell counts were performed using 0.04% trypan blue/PBS. FACS phenotyping of cell surface markers and the costimulatory molecules was performed as described previously (41).

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**FIGURE 1.** FACS phenotyping of the A20 cell line variants. Shown on the x-axis are relative fluorescence levels (FL1-H) following Ab and FITC-labeled second-step staining; shown on the y-axis are cell counts; 10⁶ cells were analyzed in each test. Isotype control staining of the cells appears as shaded histograms, and test staining for cell surface markers appears as outlined histograms. Staining is 1 μg of Ab per 10⁶ cells. Similar phenotypic analyses were performed before every experimental assay.
Animal studies
Female BALB/c and C57BL/6 mice age 8–10 wk were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). All mice were maintained in accordance with University of Toronto guidelines and following the approval of independently reviewed protocols by the Animal Facility Committee at the University of Toronto. Mice were sacrificed when the tumors reached 2 cm in diameter.

Allogeneic MLR assays
MLR assays were performed as described previously (30). Irradiated A20 tumor variants were plated at 2 × 10^5 cells per well along with 2 × 10^6 C57BL/6 T cells in 96-well plates (Nalge Nunc International, Rochester, NY). Cells were incubated for 3 days before measurement of IL-2 and 5 days before the measurement of IL-4 using bioassays.

Bioassays to measure the levels of biologically active IL-2 or IL-4
MLR supernatants were harvested and levels of biologically active IL-2 and IL-4 were measured in bioassays as described elsewhere (41).

Tumor implantation and growth
Tumor variants were washed free of serum with RPMI 1640 and 10^6 cells implanted s.c. into the right flank of naive BALB/c mice. Surviving mice, in which no tumor formed, were challenged by s.c. injection in the left flank. Mice were checked for tumor formation at least three times weekly throughout the experimental period, with daily checks at times of peak tumor incidence.

CTL assays
Mice that survived injection with tumor variants and challenge with the parental tumor line were sacrificed and their spleens removed. CTL assays were performed as described previously (30). For Ab blocking experiments, cells were incubated in the presence of 20 μg/ml Ab during the effector phase.

Results
Generation and phenotypic analysis of A20 variants expressing 4-1BBL
A20 variants derived from independent clones expressing elevated levels of 4-1BBL were generated as described in Materials and Methods. Two A20 variants expressing high levels of 4-1BBL were selected for further study and compared with the A20 parental line, A20 transfected with vector alone (A20/CMV), A20 transfected with B7-1 (A20/B7-1), and A20 transfected with B7-2 (A20/B7-2) (Fig. 1). Weekly FACS phenotyping indicated that the expression of the costimulatory molecules and phenotypic markers were stable. Cell cycle analyses and 4-1BBL expression were simultaneously investigated by propidium iodide staining and FACS and indicated no cell cycle variation of 4-1BBL expression in the parental A20 cells or the A20/4-1BBL clones. Furthermore, the cell lines showed no variation in growth rates as determined by daily cell counts of plated cells (data not shown). The A20 variants showed similar levels of IgG, MHC I, and MHC II expression. As expected, the parental A20 cell line expressed B7-2 but undetectable levels of B7-1 and 4-1BBL. A20 transfected with the vector control showed a slight elevation of B7-1 and 4-1BBL, and this is attributed to clonal variation during the selection process. The A20/B7-1 variant showed elevated B7-1 and levels of B7-2 similar to those of the parental line. Surprisingly, the A20/B7-2 line also showed enhanced 4-1BBL expression, and the A20/4-1BBL clones showed enhanced B7-1 and B7-2 expression. However, the A20/4-1BBL line showed the highest level of 4-1BBL of all the variants. We attribute the acquisition of 4-1BBL on the B7-2 transfectant and B7-1 on the 4-1BBL transfectant to the cloning/selection process. Nevertheless, the panel of transfectants described in Fig. 1 provides a useful set of variants for assessing the impact of 4-1BBL on the tumorigenicity of the A20 lymphoma.

MLRs indicate that B7-1 induces an IL-2 and IL-4 response by allogeneic CD4+ T cells, while all other variants do not
We performed MLR to determine the response of allogeneic T cells to the A20 cell line variants. Using bioassays we found that the responding T cells produced biologically active IL-2 and IL-4 in response to highly elevated expression of B7-1 (A20/B7-1) (Fig. 2, A and B). However, no detectable IL-2 or IL-4 was produced by the responding T cells to the parental or vector controls or to the A20/B7-2 or A20/4-1BBL clones.

Tumor variants expressing B7-2 and 4-1BBL are immunogenic and protect against further systemic challenge with unmodified parental A20 cells
Injections of 10^6 A20, A20/CMV vector control, or A20/B7-1 cells in the right flank of the BALB/c mice resulted in similar rates of
tumor formation and were highly tumorigenic despite the expression of some level of B7-2 on the cell surface (Fig. 3A). None of the mice injected with either of the A20/4-1BBL clones (expressing the highest level of 4-1BBL on the cells surface) developed tumors in the 150-day follow-up period in any of the experiments. Mice injected with A20/B7-2 cells that expressed elevated levels of 4-1BBL (but not to the extent of the A20/4-1BBL variant) showed a tumor development frequency of 25% compared with 100% in mice injected with the A20 parental line. 81.25% in mice injected with the A20/CMV control, and 93.75% in mice injected with A20/B7-1. In addition, tumors that formed in mice injected with A20/B7-2 developed at a later time point (Fig. 3A). FACS analysis of three of four of these tumors indicated that 4-1BBL expression had been completely down-regulated compared with the original A20/B7-2 clone injected into these mice and in two of three of the tumors B7-2 had also been fully down-regulated (Fig. 4).

Mice surviving the primary injection with the A20 variants were challenged on the opposite flank with the parental A20 line. All but two mice resisted this systemic challenge, one originally injected with A20/B7-2 (1 of 16) and one originally injected with A20/4-1BBL (1 of 32). To control for the tumorigenicity of these A20 parental cells used for the challenge, naive BALB/c mice were also injected with the same cells. The naive mice all formed tumors on the left flank within 25 days of injection (Fig. 3B). To ensure that the A20/B7-2 and A20/4-1BBL clones were all potentially tumorigenic in the absence of an intact immune system, nude mice were injected with $10^6$ cells of A20/B7-2, A20/B7-2, or A20/4-1BBL clones 1 or 2. All of these mice formed tumors with no variation in tumor development rates (Fig. 3C).

Mice injected with tumor variants expressing B7-2 or 4-1BBL showed ex vivo CTL activity against the parental tumor A20 and K46J following secondary restimulation in vitro

Mice injected with the A20 cell line variants were analyzed at days 9, 15, and 21 postinjection for CTL activity against the A20 parental clone following in vitro stimulation with irradiated A20. No CTL activity against A20 was observed except in one of three mice injected with A20/B7-2 and one of three mice injected with A20/4-1BBL at days 15 and 21 (data not shown). Mice that had resisted tumor challenge were sacrificed at greater than day 150 and analyzed for CTL activity against A20 and a panel of cell line controls following ex vivo stimulation of the splenocytes using irradiated parental A20 cells. CTL activity against A20 and another BALB/c lymphoma, K46J, was observed in splenocytes from mice injected with A20/B7-2 and both clones of A20/4-1BBL (Fig. 5, A and B). A small amount of background CTL activity was seen against the SCID thymoma ST-D2 and the C57BL/6 lymphoma EL4 cell lines by splenocytes from mice in these groups.

CTL activity was mediated by B7-1, B7-2, and 4-1BBL interactions

To test the importance of the different costimulatory ligands in CTL activation, soluble 4-1BB receptor (4-1BB-AP) or anti-B7-1 or anti-B7-2 was added during the in vitro secondary re-activation as described in Materials and Methods. Splenocytes from mice immunized with A20/B7-2 showed diminished CTL activity following blocking with anti-B7-1 and anti-B7-2 but not anti-B7-2 alone. However, CTL activity was further blocked in the presence of a combination of anti-B7-1, anti-B7-2, and 4-1BB-AP (Fig. 6A). Splenocytes from mice immunized with A20/4-1BBL showed reduced CTL activity in the presence of the combination of anti-B7-1, anti-B7-2 and 4-1BB-AP but not when any of the other Ab and 4-1BB-AP combinations were used (Fig. 6B). Cells only, Ab, and AP controls were also used as negative controls for blocking reagents, and ST-D2 was used as a control for background lysis. The data show that in splenocytes from mice immunized with A20/B7-2...
or A20/4-1BBL, combinations of B7-1, B7-2, and 4-1BBL signaling were all contributing factors in the induction of CTL activity against the parental tumor.

Discussion

In this study, we have shown that neither B7-2 on the A20 parental cells nor B7-1 on A20/B7-1 variants were able to mediate long-term antitumor immunity. However, expression of transfected 4-1BBL in conjunction with elevated B7-1 and B7-2 levels on the A20/4-1BBL variants resulted in long-term immunity. None of the mice injected with $10^6$ A20 variants expressing transfected 4-1BBL developed tumors in a 150-day follow-up period ($n = 32$), while all mice injected with the A20 parental or vector control cells formed tumors within 25 days ($n = 32$). In contrast, 25% of mice injected with A20/B7-2 succumbed to tumor development, albeit at a delayed time point ($n = 4$) compared with the controls. Mice that resisted tumor formation following injection with A20/4-1BBL or A20/B7-2 demonstrated long-term immunity against later systemic challenge with the A20 parental cell line. This immunity lasted at least 150 days, at which time the mice were sacrificed for CTL analyses. Our tumorigenicity studies have clearly demonstrated that 4-1BBL enhances the capacity of elevated B7-1 and B7-2 in converting this tumorigenic cell line into a long-lasting anticancer vaccine.

Only two other reports have investigated the role of the 4-1BB:4-1BBL interaction in tumor immunogenicity. Melero et al. (38) showed that administration of anti-4-1BB mAbs into DBA/2 mice following the injection of the P815 mastocytoma or Ag104A sarcoma cell line prevented the development of tumors in most mice. This tumor destruction was shown to be long lasting and specific against the parental tumor. The treatment was shown to lead to the proliferation of both CD4$^+$ and CD8$^+$ cells ex vivo, and the role of both populations of T cells in the antitumor response was confirmed by anti-CD4 and anti-CD8 depletion studies in vivo. Melero et al. (39) showed in subsequent studies that P815 tumor cells expressing transfected 4-1BBL did not form tumors in the majority of mice and that these cells conferred long-term immunity against later challenge with the wild-type tumor. CD4$^+$ and CD8$^+$ depletion studies indicated that CD8$^+$ but not CD4$^+$ cells were responsible for the immunogenicity observed in vivo. We have extended the above studies by analyzing the effects of 4-1BBL in conferring antitumor immunity to the A20 tumor cell line in the presence of the B7-1 or B7-2 molecules. We have shown that the long-term immunity conferred to the mice by the variants was dependent on an intact immune system. Tumor destruction appeared to be mediated by T cells as shown by experiments using nude mice. Although we did not demonstrate any T helper activity induced by the A20/B7-2 or A20/4-1BBL variants in MLR assays, significant CTL activity against the parental line was observed in T cells from mice that had rejected A20 challenge following A20/B7-2 and A20/4-1BBL injections. The development of CTL effectors was stimulated synergistically...
through B7-1, B7-2, and 4-1BBL signaling pathways as demonstrated by blocking experiments.

Tumors that formed in mice injected with A20/B7-2 all showed a down-regulation of B7-1, B7-2, and 4-1BBL. Ab blocking during the in vitro reactivation of CTLs further indicated that in mice injected with either A20/B7-2 or A20/4-1BBL, it was a synergy between B7-1, B7-2, and 4-1BBL signaling that played a role in the secondary reactivation of CTLs against the parental tumor ex vivo. 4-1BBL is thought to enhance the effects of B7-1 and B7-2 levels and has been shown to prolong T cell activation when CD28 unresponsiveness may otherwise develop (45). Our data suggest a close association between B7-1, B7-2, and 4-1BBL expression and antitumor immunity in this model system.

A20 cells transfected with 4-1BBL were only able to form tumors in the absence of an intact immune system, as seen following the injection of these tumor cells into BALB/c nu/nu mice. Other studies have shown that 4-1BBL may stimulate naive T cells to secrete IL-2 (23, 24, 30, 42, 46) and IFN-γ (46) in MLR assays. However, in our MLR assays, only the A20/B7-1 variant elicited an IL-2 and IL-4 cytokine response from naive allogeneic T cells. The level of cytokine expression induced by the A20/B7-1 cells appears to have been below the level necessary for an antitumor response in syngeneic mice, as these cells showed no delay in tumor formation in mice compared with vector and parental cell line controls. However, in vitro stimulated splenocytes from mice injected with A20/B7-2 (A) or A20/4-1BBL (B) were incubated with chromium-labeled A20 parental cells, and lytic activity was determined as a percentage of total lysis following adjustments for spontaneous lysis. Chromium-labeled ST-D2 cells were used as a control for specific lysis of the target cell population. Data are representative of at least two mice assessed with all Ab combinations and controls in two independent experiments.
tumor-associated Ag. Only background levels of CTL activity were seen against the congeneric BALb/c SCID thymoma line ST-D2 or the allogeneic C57BL/6 lymphoma cell line EL4, suggesting that the observed lysis of A20 and K46l was specific and not due to contaminating NK activity. No difference in the level of CTL activity against the parental tumor was observed between mice injected with A20/B7-2 or either of the A20/4-1BBL clones, and very low CTL activity was observed against any of the tumor cell lines by splenocytes from naive mice.

Future studies in other tumor models should help determine whether the 4-1BBL globally enhances antitumor immunity in combination with the B7 molecules or whether this capacity varies with tumor type and the immunogenicity of the tumor line. We have shown that in this B cell lymphoma model, 4-1BBL has converted the tumorigenic A20 cell line into a potent and long-lasting antitumor vaccine. We have demonstrated antitumor immunogenicity protective not only against systemic wild-type tumor challenge with the parental cell line in vivo but immunogenicity that may also lead to the development of CTLs able to kill cells from a syngeneic tumor cell line in vitro. The tumor destruction observed in this study appeared to be CD8+ mediated in the absence of any detectable CD4+ activity in the MLR, and it would appear to occur through both the CD28 and 4-1BB signaling pathways on responding T cells. This study indicates the complexity of the signaling interactions between these costimulatory signals and highlights the importance of the 4-1BBL molecule in antitumor responses.

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