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Tumor Rejection After Direct Costimulation of CD8$^+$ T Cells by B7-Transfected Melanoma Cells

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A variety of tumors are potentially immunogenic but do not stimulate an effective anti-tumor immune response in vivo. Tumors may be capable of delivering antigen-specific signals to T cells, but may not deliver the costimulatory signals necessary for full activation of T cells. Expression of the costimulatory ligand B7 on melanoma cells was found to induce the rejection of a murine melanoma in vivo. This rejection was mediated by CD8$^+$ T cells; CD4$^+$ T cells were not required. These results suggest that B7 expression renders tumor cells capable of effective antigen presentation, leading to their eradication in vivo.

T cells recognize processed peptide antigens complexed with molecules of the major histocompatibility complex (MHC) (*1–4*). However, recognition of antigen by the T cell antigen receptor (TCR) is not sufficient for activation; a second costimulatory signal is required (*5*). The costimulation results from an interaction of the CD28 molecule on the T cell surface with its ligand, B7, on the surface of an antigen-presenting cell (APC) (*6–12*). The expression of B7 is generally limited to "professional" APCs: macrophages, dendritic cells, and activated B cells (*7, 13, 14*). Most epithelial cells do not express B7. One possibility to account for the tumorigenicity of MHC-expressing tumors is that despite presentation of potentially immunogenic peptides in the context of MHC molecules, tumors may lack the costimulatory molecule, B7, and thus fail to elicit an effective anti-tumor T cell response (*15–20*). We now show that provision of costimulatory activity can lead to tumor rejection in vivo, and that this strategy bypasses the need for exogenous help from CD4$^+$ T cells by directly activating CD8$^+$ T cells.

The murine melanoma cell line K1735 expresses both MHC class I and class II molecules and stimulates a specific, but ineffective, immune response in vivo (*21, 22*). K1735 cells did not provide costimulation to T cells in a standard in vitro costimulation assay (below). In initial experiments to test the importance of costimulation in the generation of an anti-tumor response, monoclonal antibody (MAb) to CD28 was used as an exogenous source of costimulatory activity (*23*). Fragments of K1735 solid tumors raised in athymic mice were implanted subcutaneously (s.c.) into syngeneic C3H/HeN mice. The mice were treated with repeated intraperitoneal injections of MAb to CD28 (ascites). Treatment with anti-CD28 slowed the growth of the melanoma (Fig. 1). Although complete

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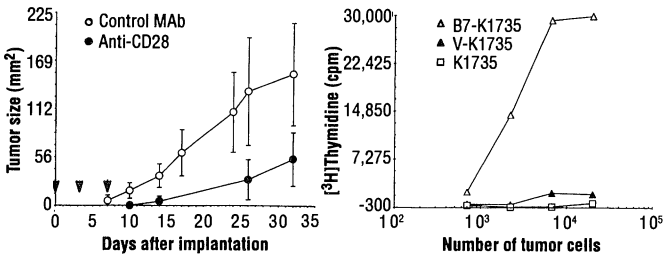

Fig. 1 (left). Treatment with anti-CD28 slowed the growth of a melanoma in vivo. A solid tumor of the K1735 melanoma was raised in a C3H/HeN mouse (Fredrick Cancer Research Center, NCI-NIH). Two fragments (~1 mm^3 each) of this tumor were implanted s.c. in the backs of groups of five female C3H/HeN mice, 8 to 10 weeks old. These mice received six 50-μl intraperitoneal injections of either anti-CD28 ascites or an irrelevant hamster ascites as indicated by arrows. Bisecting tumor diameters were measured blindly on coded treatment groups. The final tumor size of euthanized mice, to a maximum of 200 mm^2, was included in data calculation for subsequent time points. Error bars represent standard deviation. Experiment shown is representative of two separate experiments, using a total of ten mice in each treatment group. **Fig. 2 (right).** B7-K1735 provided costimulation to T cells in vitro. K1735 cells were transfected by electroporation with an expression vector (*35*) containing cDNA encoding murine B7 (*7*) or with the vector alone. Drug-resistant cells were cloned by limiting dilution and surface expression of B7 on expanded clones was verified by flow cytometry, using the B7-binding fusion protein, CTLA4-Ig (*36*). Lymph node T cells from C3H/HeN mice were partially purified by panning twice on plastic plates coated with goat antibody to mouse immunoglobulin, then cultured in complete media (RPMI, supplements, and antibiotics) at 37°C on plates coated with a suboptimal concentration (1 μg/ml) of anti-CD3 (MAb 500A2) in the presence of titrated numbers of irradiated B7-K1735, V-K1735, or K1735 cells. Cultures were pulsed with [^{3}H]thymidine (Amersham) after 48 hours and harvested after 18 hours. Proliferation in response to anti-CD3 alone was 549 cpm. Proliferation of T cells and tumor cells in the absence of anti-CD3 has been subtracted from presented values. Values represent averages of triplicate cultures that varied by no more than 25%.

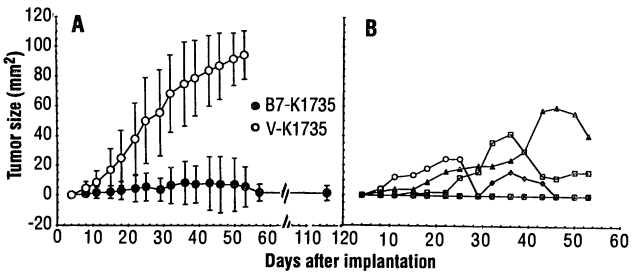

Fig. 3. Growth of K1735 transfectants in C3H/HeN mice. Solid tumors were raised in BALB/c nude mice (Simonsen, Gilroy, California) from cell suspensions and maintained by serial passage. Two tumor fragments (~1 mm^3) were implanted s.c. in the backs of groups of ten C3H/HeN mice. (**A**) Average tumor size of B7-K1735 and V-K1735 implanted mice. Error bars represent standard deviation. (**B**) Growth of ten individual B7-K1735 tumors; six of the ten tumors did not grow. The final tumor size of euthanized mice, to a maximum of 100 mm^2, was included in data calculation for subsequent time points. Data shown are representative of three separate experiments; a total of 25 mice implanted with each tumor were used.

rejection was not obtained, these results suggest that provision of costimulation can enhance an anti-tumor immune response.

In order to confer costimulatory capacity, we transfected K1735 cells with an expression vector containing murine cDNA encoding B7 [B7-K1735;(7)] or with the vector alone (V-K1735). The ability of B7-K1735 cells or V-K1735 cells to provide costimulation to T cells stimulated with MAb to CD3 was tested in vitro in a standard costimulation assay. The B7-expressing transfectant, but neither the control transfectant nor the parental K1735 cells, was effective in providing costimulation to T cells (Fig. 2).

Both B7-K1735 and V-K1735 tumors were equally tumorigenic in athymic nude mice, demonstrating that these lines are not growth-impaired in vivo in the absence of T cells. However, in intact syngeneic C3H/HeN mice, fragments of V-K1735 tumors grew aggressively, whereas B7-K1735 tumor fragments grew poorly (Fig. 3A). By day 38, 50% of the mice bearing V-K1735 tumors were euthanized because of large or ulcerated tumors, and all mice were euthanized by day 53. In contrast, after 120 days, all mice implanted with B7-K1735 appeared healthy and all tumors had either disappeared (9/10) or become static (1/10). In the minority of mice in which B7-K1735 tumors appeared, the tumors regressed over time (Fig. 3B); this suggests that an active process in vivo prevents sustained growth in C3H mice.

We next sought to determine whether rejection of B7-K1735 resulted in protection against subsequent challenge with the B7-negative tumor (V-K1735). C3H/HeN mice were implanted with B7-K1735 fragments and on day 25 after implantation were challenged subcutaneously with fragments of V-K1735. Prior exposure to B7-K1735 protected 89% of mice against

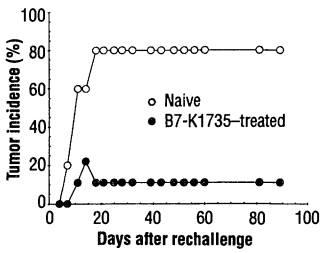

Fig. 4. Exposure to B7-K1735 protected against subsequent challenge with V-K1735. C3H/HeN mice were implanted s.c. with fragments of B7-K1735. On day 25 after implantation, nine treated and five naive mice were implanted s.c. in the back at a distant location with fragments of V-K1735. Data shown are representative of two experiments; a total of 19 mice implanted with B7-K1735 were used.

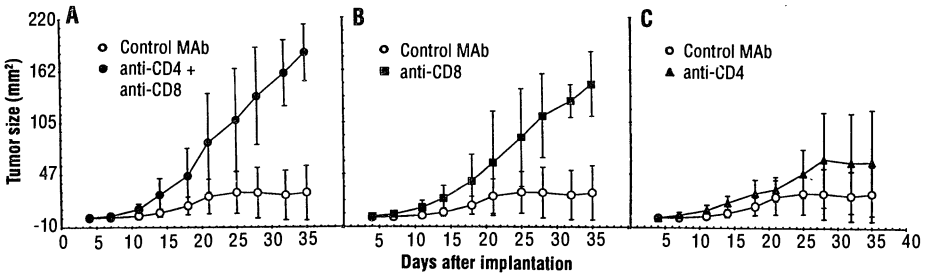

Fig. 5. Growth of B7-K1735 in mice depleted of T cell subsets. (**A**) Average tumor size in mice treated with both anti-CD4 and anti-CD8 (eight mice) or a control antibody (ten mice). (**B**) Average tumor size in mice treated with anti-CD8 (ten mice). (**C**) Average tumor size in mice treated with anti-CD4 (eight mice). C3H/HeN mice were injected on three successive days with ammonium sulfate–purified ascites preparations of anti-CD4 (MAb GK1.5, 0.2 mg per injection), anti-CD8 (MAb 53.672, 0.5 mg per injection), a combination of anti-CD4 and anti-CD8 (total of 0.7 mg per injection), or an isotype-matched, nonreactive control (MAb 2.43, 0.7 mg per injection). Three days after the last injection, depletion of each subset (>99%) was verified by flow cytometry analysis of peripheral blood lymphocytes. Fragments of B7-K1735 were implanted s.c. and antibody injections were continued every 4 to 7 days. Error bars represent standard deviation.

growth of V-K1735 for 90 days, whereas tumors appeared in 80% of naïve mice by day 18 (Fig. 4). Thus, B7-K1735 can effectively prime for a response against challenge by the control V-K1735, which demonstrates that B7-K1735 and V-K1735 are immunologically cross-reactive. This supports the notion that the costimulatory activity provided by the B7 gene product is the functionally important difference between these tumors.

In order to directly test whether $CD4^+$ T cells, $CD8^+$ T cells, or both subsets were responsible for the rejection of B7-K1735, mice were depleted of $CD4^+$ cells, $CD8^+$ cells, or both, by antibody treatments in vivo before implanting B7-K1735. As expected, B7-K1735 tumors grew aggressively in mice depleted of both $CD4^+$ and $CD8^+$ T cells, demonstrating that T cells are responsible for rejection of B7-K1735 in intact mice (Fig. 5A). Growth of B7-K1735 was greatly enhanced in CD8-depleted mice relative to nondepleted mice, demonstrating that $CD8^+$ cells are required for the rejection of B7-K1735 (Fig. 5B). In contrast, tumor growth was only slightly enhanced in CD4-depleted mice, demonstrating that $CD4^+$ cells are not necessary for rejection (Fig. 5C). These results demonstrate that "help" from $CD4^+$ cells does not seem to be necessary for the induction of $CD8^+$ activity in this system, and that $CD8^+$ cells are primarily responsible for the rejection of B7-K1735.

Exogenous help by $CD4^+$ T cells can augment a cytotoxic T lymphocyte (CTL) response (*24–27*). However, effector $CD8^+$ CTL can be generated in the absence of $CD4^+$ cells, provided that accessory cells are present (*28–31*). We have found that costimulation provided by the B7-CD28 interaction allows the generation of allogeneic CTL from naïve precursors in the absence of $CD4^+$ cells, and that costimulation is not required for effector function (*12*). The results presented here are consistent with these observations. The lack of growth of the B7-K1735 tumor in CD4-depleted mice and the growth in CD8-depleted mice suggests that B7 can directly costimulate $CD8^+$ T cells in vivo, independent of the presence of $CD4^+$ T cells. In addition, the protection conferred by the B7-K1735 tumor to subsequent challenge by V-K1735 suggests that the expression of B7 is not necessary for killing in vivo.

We have shown that provision of costimulation by surface expression of B7 provides protection against the growth of a melanoma in vivo. This protection is mediated primarily by $CD8^+$ T cells, which can function in the absence of help from $CD4^+$ cells. Other studies have focused on boosting anti-tumor immune responses by providing "help" in the form of lymphokines normally produced by $CD4^+$ T cells (*32–34*). Manipulation of costimulation offers another strategy for tumor immunotherapy. Direct activation of CTL by tumors with appropriate costimulation may "jump-start" the anti-tumor response by bypassing the need for exogenous help, which could lead to a faster, more effective anti-tumor response in situ. This may provide a means to elicit effective CTL responses to established tumors in the immunotherapy of primary or metastatic disease.

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Cyclic ADP–Ribose in Insulin Secretion from Pancreatic β Cells

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Inositol 1,4,5-trisphosphate (IP_3) is thought to be a second messenger for intracellular calcium mobilization. However, in a cell-free system of islet microsomes, cyclic adenosine diphosphate–ribose (cADP-ribose), a nicotinamide adenine dinucleotide (NAD^+) metabolite, but not IP_3, induced calcium release. In digitonin-permeabilized islets, cADP-ribose and calcium, but not IP_3, induced insulin secretion. Islet microsomes released calcium when combined with the extract from intact islets that had been incubated with high concentrations of glucose. Sequential additions of cADP-ribose inhibited the calcium release response to extracts from islets treated with high concentrations of glucose. Conversely, repeated additions of the islet extract inhibited the calcium release response to a subsequent addition of cADP-ribose. These results suggest that cADP-ribose is a mediator of calcium release from islet microsomes and may be generated in islets by glucose stimulation, serving as a second messenger for calcium mobilization in the endoplasmic reticulum.

Glucose is the primary stimulus of insulin secretion and synthesis from the pancreatic islets of Langerhans (*1*, *2*). Increases in intracellular Ca^{2+} concentration mediate the biochemical events that couple glucose stimulation to insulin secretion by the islets, and mobilization of Ca^{2+} from intracellular stores in the endoplasmic reticulum is important in this process (*3*). It has been thought that IP_3 is a second messenger for Ca^{2+} mobilization from intracellular stores (*4*). However, some aspects of agonist ac-

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