A Reassessment of The Role of B7-1 Expression in Tumor Rejection

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Summary

Introduction of the *B7-1* gene into murine tumor cells can result in rejection of the B7-1 transductants and, in some cases, systemic immunity to subsequent challenge with the nontransduced tumor cells. These effects have been largely attributed to the function of B7-1 as a costimulator in directly activating tumor specific, major histocompatibility class I–restricted $CD8^+$ T cells. We examined the role of B7-1 expression in the direct rejection as well as in the induction of systemic immunity to a nonimmunogenic murine tumor. B-16 melanoma cells with high levels of B7-1 expression did not grow in C57BL/6 recipient mice, while wild-type B-16 cells and cells with low B7-1 expression grew progressively within 21 d. In mixing experiments with B7-1^{hi} and wild-type B-16 cells, tumors grew out in vivo even when a minority of cells were B7-1^-. Furthermore, the occasional tumors that grew out after injection of 100% B-16 B7-1^{hi} cells showed markedly decreased B7-1 expression. In vivo antibody depletions showed that NK1.1 and $CD8^+$ T cells, but not $CD4^+$ T cells, were essential for the in vivo rejection of tumors. Animals that rejected B-16 B7-1^{hi} tumors did not develop enhanced systemic immunity against challenge with wild-type B-16 cells. These results suggest that a major role of B7-1 expression by tumors is to mediate direct recognition and killing by natural killer cells. With an intrinsically nonimmunogenic tumor, this direct killing does not lead to enhanced systemic immunity.

Experimentally induced animal tumors can be classified as immunogenic and nonimmunogenic (1). Immunogenic tumors are rejected after transplantation into syngeneic animals that had been previously immunized with irradiated cells of the same tumor. Nonimmunogenic tumors are not rejected when similarly tested.

Evidence suggests that rejection of immunogenic tumors is primarily mediated by T cells (1), with both $CD8^+$ CTL and $CD4^+$ Th cells playing important roles (2, 3). In general, the induction and activation of T cells requires two signals from APC (for reviews see references 4–6). The first signal, the engagement of the TCR to its cognate peptide–MHC ligand, provides specificity. The second signal is provided by costimulatory molecules expressed on APCs binding to their counterreceptors on the T lymphocyte (4). Binding of the TCR with peptide–MHC complexes in the absence of costimulation results in T cell inactivation or "anergy," which is associated with a block of IL-2 gene transcription (7).

Among the known costimulatory molecules, the B7 family of membrane proteins appears to be the most potent. B7 is a member of the Ig superfamily (8), and it is expressed on the majority of APCs, such as dendritic cells, activated macrophages, and activated B cells. The B7 costimulatory pathway involves at least two molecules, B7-1 (CD80) (8) and B7-2 (CD86) (9–11). Both B7-1 and B7-2 can interact with their counterreceptors, CD28 and CTLA-4, on T cells (10–12). The importance of B7-1 as a costimulator involved in generating an antitumor immune response has been suggested by a number of in vivo experimental systems (13–15). In these studies, B7-1–expressing tumor cells are rejected in syngeneic hosts, whereas unmodified tumor cells are not. These studies suggest that 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. The rejection of B7-1–expressing tumors has been proposed to be mediated by $CD8^+$ T cells (13, 15) or $CD4^+$ T cells (14).

The binding of the TCR to its peptide–MHC ligand may be insufficient to activate T cells against nonimmunogenic tumors such as B16 murine melanoma cells since MHC class I expression may be low in these tumors. Consequently, generation of specific CTL activity may be inadequate as compared to that by other high MHC class I–expressing tumors. It is not clear, however, whether enhanced second signal such as increased expression of B7-1 costimulatory molecules expressed on APCs can compensate for the deficiency of first signal. In this study, we have chosen B16 as a

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model nonimmunogenic tumor. We have investigated the effect of both the B7-1 level and the quantity of high B7-1–expressing tumor cells required to cause the rejection of nonimmunogenic tumor cells. Furthermore, we explored the role of lymphocyte subsets in the rejection of high B7-1–expressing tumor cells. Our results suggest that both the presence and levels of B7-1 expression on B7-1–transduced B16 cells can affect tumor growth in vivo. Mixing experiments with B7-1^{hi} and B7-1^{-} B-16 cells further indicated that B7-1 expression mediates direct killing. In addition, NK cells and $CD8^+$ cells, but not $CD4^+$ T cells, are essential for the in vivo rejection of high B7-1–expressing nonimmunogenic tumor cells.

Materials and Methods

Tumor Cells. The F10 subline of B-16 melanoma cells was obtained from the National Institutes of Health, Division of Cancer Therapy tumor repository. Tumor cells were cultured in vitro in RPMI 1640 media, supplemented with 10% FCS, penicillin/streptomycin (50 U/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), and nonessential amino acids (0.1 mM), and they were grown at 37°C, 5% CO_2. All cells were periodically tested for and found to be free of mycoplasma contamination.

Transduction of B-16 Cells with B7-1 Molecule Using Retroviral Vector. Transduction of murine *B7-1* gene into B-16 was performed with the MFG retroviral vector system as described previously (16). Briefly, in this vector, Moloney murine leukemia virus long terminal repeat sequences are used to generate both a full-length viral RNA (for encapsidation into virus particles) and a subgenomic mRNA (analogous to the Moloney murine leukemia virus env mRNA) that is responsible for the expression of inserted sequences. The B7-1 gene–containing MFG were introduced into CRIP cells to a generate recombinant virus with an amphotropic host range. Transduction was performed as described previously (16). After transduction, the retroviral supernatant was removed, and the cells were grown in culture for an additional 3 d to allow for integration and expression of the transferred DNA. The expression of B7-1 was demonstrated by flow cytometry using CTLA-4Ig, a soluble genetic fusion between the extracellular domain of CTLA-4 and an IgC γ chain (12), and goat anti–human IgG_1-FITC (Cappel Laboratories, Cochranville, PA).

Tumor Growth Experiments. The B-16 B7-1^+ cells are cloned by limiting dilution for different levels of B7-1 expression as determined by flow cytometry. Live B-16 B7-1^+ tumor cells with different levels of B7-1 expression and wild-type B-16 cells were injected into 10 C57 BL/6 mice subcutaneously at a dose of 10^6 cells per mouse. In the second experiment, high B7-1–expressing B-16 tumor cells are mixed with wild-type B-16 cells in different proportions (0, 25, 50, 75, and 100%). Groups of eight C57BL/6 mice were injected subcutaneously with a final dose of 10^6 cells per mouse. Tumor growth was measured regularly. Mice were immediately euthanized when tumor growth was observed.

In Vivo Antibody Depletion Experiments. In vivo antibody depletions have been described previously (17). Briefly, depletions were started 1 wk before tumor inoculation. 10 C57BL/6 mice were used in each group. mAb GK1.5 (18) was used for CD4 depletions, mAb 2.43 (19) was used for CD8 depletions, and mAb PK136 (20) was used for NK1.1 depletion. Ammonium sulfate-purified ascites fluid (titered at >1:2,000 by staining of splenocytes on the FACS® [Becton Dickinson & Co., Mountain View, CA]) were injected intraperitoneally (0.1 ml per mouse) every other day for the 1st wk and once per week after inoculation. Depletion of lymphocyte subsets was assessed on the day of live tumor injection, and weekly thereafter by flow cytometric analysis of spleen cells stained with 2.43 or GK1.5 followed by FITC-labeled goat antibody to rat IgG (Southern Biotechnology Associates, Birmingham, AL), or PK136 followed by FITC-labeled goat antibody to mouse IgG. For each time point of analysis, >99% depletion of the appropriate subset was achieved with normal levels of the other subsets. Depletion was terminated on the 21st d after tumor inoculation.

In Vivo Protection Assays. Cells for injection were harvested from in vitro culture by trypsinization after limited expansion and washed three times in serum-free HBSS. For vaccination, 1 million live B7-1^{hi} B-16 cells were injected into each C57BL/6 mouse. For challenge, various doses of wild-type B-16 tumor cells (10^6, 10^5, and 10^4) were injected 2 wk after vaccination. All injections were in a volume of 0.1 ml given subcutaneously in the left (vaccine) or right (challenge) flank. All experiments included 10 mice per group, and each experiment was repeated at least once. Mice were monitored twice per week and killed after the tumor development.

Results

Level of B7-1 Expression Affects Tumor Growth. To generate B7-1–expressing B-16 cells, we used the MFG retroviral vector system to transduce the murine *B7-1* gene into B-16 cells. Cells with different levels of B7-1 expression were obtained from clones generated through limiting dilution, with B7-1 levels determined by flow cytometry. As shown in Fig. 1, two independent B-16 B7-1^+ clones revealed different levels of B7-1 expression. These cell lines exhibited similar growth kinetics in vitro as well as in SCID mice in vivo (data not shown). When the same number of tumor cells (10^6 per mouse) were injected subcutaneously into C57BL/6 mice, however, cells with high levels of B7-1 expression failed to grow in any of the recipient mice (Fig. 2, *open squares*). In comparison, low B7-1–expressing cells (Fig. 2, *closed squares*) or wild type B-16 cells (Fig. 2, *closed circle*) continued to grow in all of the injected mice. Tumor growth in all these mice was progressive without any plateau or regression at later time points. These results suggest that only high levels of B7-1 expression on nonimmunogenic tumor cells can lead to their rejection in vivo.

Outgrowth of B7-1^- Tumor Cells in a Mixed Population. Because of the documented role of B7-1 as a costimulator in the initiation of T cell activation, the immunologic effects of B7-1–transduced tumors have been largely attributed to the enhancement of priming of tumor-specific $CD8^+$ CTL precursors. If this form of costimulatory activity represented the major effect of high B7-1 expression, not every cell would need to express B7-1 to activate $CD8^+$ responses. If B7-1 mediated direct target recognition, however, then even a minority of B7-1^- cells in the population would result in tumor formation. This distinction is most easily made with mixing experiments. We mixed B7-1^{hi}–expressing B-16 tumor cells with wild-type

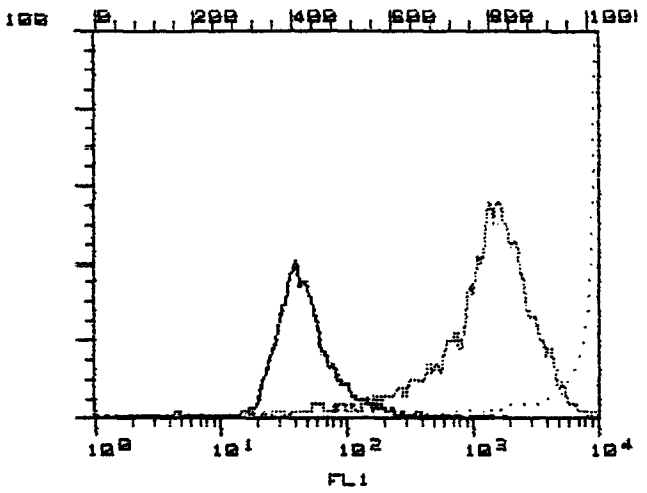

Figure 1. Variation of B7-1 expression in different clones by flow cytometry. Individual clones of B-16 B7-1$^+$ cells were isolated by limiting dilution. The level of B7-1 expression was determined by flow cytometry with CTLA-4Ig followed by goat anti-human IgG_1-FITC (Cappel Laboratories). MC1 and MC2 represent two different clones of B7-1–expressing B-16 cells. In contrast to wild-type B-16 cells (———), MC1 (. . . . .) showed the highest level of B7-1 expression, while MC2 (.....) displayed an intermediate level of B7-1 expression. Staining of wild-type B-16 cells with CTLA-4Ig followed by goat anti-human IgG_1-FITC was similar to that with goat anti-human IgG_1-FITC alone.

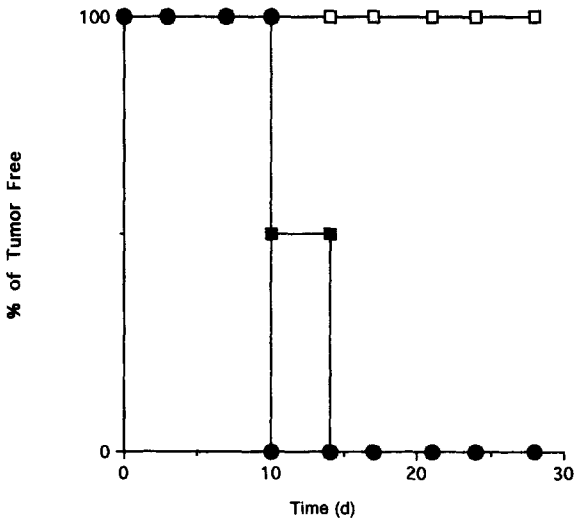

Figure 2. Level of B7-1 expression affects tumor growth in vivo. Live MC1 (*open squares*), MC2 (*closed squares*), and wild-type B-16 (*closed circle*) tumor cells were injected into groups of 10 C57 BL/6 mice subcutaneously at a dose of 10^6 cells per mouse. The mice were monitored for evidence of tumor growth by palpation and inspection twice per week. –□–, B16 B7-1 (MC1); –●–, B16 wild type; –■–, B16 B7-1 (MC2).

B-16 cells in different proportions (0, 25, 50, 75, and 100%) immediately before subcutaneous injection. Each C57BL/6 mouse was injected with a total dose of 10^6 cells. Fig. 3 shows the results of tumor growth in each group of mice. Tumors grew within 3 wk in all of the mice injected with 0, 25, 50, and 75% B7-1^{hi}–expressing B-16 cells. Again, all tumor growth was progressive. In a repeat of the experiment shown in Fig. 2, tumors grew out in two of eight mice injected with B-16 B7-1^{hi} cells. When the explanted tumor cells were stained for B7-1, decreased B7-1 expression comparable to that in the B-16 B7-1^{lo} clone was noted in these tumor cells (Fig. 4). These results suggest that there is a direct killing of B7-1$^+$ tumor cells in vivo and that bystander B7-1^{hi}–expressing tumor cells do not help in the rejection of even a minority of low or negative B7-1–expressing tumors.

NK Cells and CD8$^+$ T Cells Are Essential for the Rejection of B7-1$^+$ Tumors. To determine the types of lymphocytes that are important for the rejection of B7-1$^+$ tumor cells in our in vivo model, we performed in vivo antibody depletion experiments. Depletion of lymphocyte subsets was assessed on the day of tumor injection, and weekly thereafter by flow cytometric analysis of spleen cells. More than 99% depletion of the appropriate subset was achieved with normal levels of the other subsets (data not shown). As shown in Fig. 5 *A*, all the mice with CD8$^+$ T cell or NK1.1 cell depletions failed to reject tumors. In comparison, mice with CD4$^+$ T cell depletion did not grow tumors. These

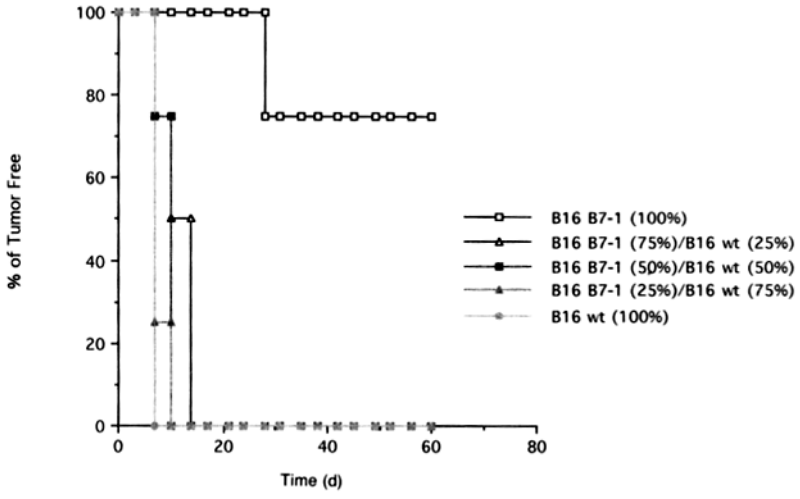

Figure 3. Outgrowth of B7-1–negative tumor cells in a mixed population. High B7-1–expressing B-16 tumor cells (MC1) were mixed with wild-type B-16 cells in different proportions (0, 25, 50, 75, and 100%). Groups of eight C57BL/6 mice were injected subcutaneously with a final dose of 10^6 cells per mouse. Tumor growth was measured as described above.

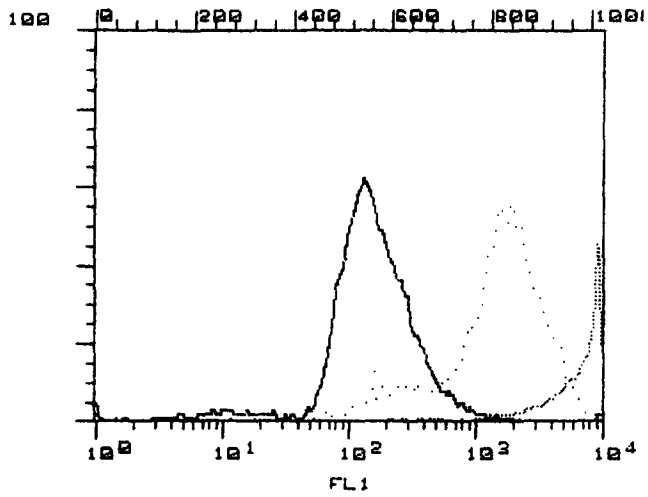

Figure 4. Decreased B7-1 expression in explanted tumors. The explant tumors were minced and digested with collagenase. Individual cells were stained with CTLA-4 Ig followed by goat anti-human IgG_1-FITC (Cappel Laboratories). B-16 B7-1 MC1 (.....) and B-16 wt (———) were used as positive and negative controls for B7-1 stainings. Explanted tumor cells (. . . . . .) were derived from mice injected with 100% of B-16 B7-1 MC1. Note the decreased level of B7-1 expression in explanted tumor cells compared to the original B-16 B7-1 (MC1) tumor cells. Staining of wild-type B-16 cells with CTLA-4 Ig followed by goat anti-human IgG_1-FITC was similar to that with goat anti-human IgG_1-FITC alone.

results suggest that $CD8^+$ T cells and NK1.1 cells, but not $CD4^+$ T cells are essential for the primary rejection of B7-1^{hi}–expressing B16 tumor cells. Furthermore, as shown in Fig. 5 *B*, 1 wk after termination of NK1.1 depletion, mice began to show a continuous regression of tumors that was complete within 10 d. In comparison, the termination of $CD8^+$ T cell depletion did not lead to the regression of tumors. These results suggest that NK1.1 cells are the most potent mediator of regression of B7-1^{hi} expressing B-16 tumor cells.

Immunization of B-16 B7-1^{hi} Tumor Cells Does Not Lead to Protection against Challenge with Wild-type B-16 Tumor Cells. We performed tumor protection experiments to investigate if B-16 B7-1^{hi} tumor cells can generate systemic immunity against challenge with wild-type B-16 tumor cells. 1 million live B7-1^{hi} B-16 tumor cells were injected subcutaneously into C57BL/6 mice for vaccination. 2 wk later, vaccinated mice were challenged with different doses of wild-type B-16 tumor cells. Our results demonstrated no protection against challenge with wild-type B-16 tumor cells (data not shown). Furthermore, introduction of the HPV-16 E7 antigen into B7-1–expressing B-16 tumor cells also did not generate systemic immunity against challenge with HPV-16 E7–expressing B-16 tumor cells (data not shown). These results suggest that a major role of B7-1 expression by tumors is to mediate direct recognition and killing by NK cells, and that this direct killing does not lead to enhanced systemic immunity with an intrinsically nonimmunogenic tumor either with or without the presence of a strong tumor rejection antigen.

Discussion

The results of these studies force a reassessment of the role of B7-1 expression by tumor cells in inducing antitumor immunity. While it has been generally accepted that B7-1 expression enhances a tumor's ability to present MHC class I–restricted antigens to $CD8^+$ T cells through costimulation, the identity of the APCs for MHC class I tumor antigens has not been experimentally determined in B7-1–transduced tumor vaccines. We have shown that for other forms of whole-cell vaccines, bone marrow–derived cells, not the tumor cell, are the exclusive APCs for MHC class I–restricted tumor antigens (21). The results presented here, indicating that high B7-1 expression by tumor cells confers direct NK and $CD8^+$ T cell–mediated killing, suggests that enhanced release of antigen by the tumor for uptake and presentation by bone marrow–derived APCs may also be an important mechanism of immune response generation by B7-1–transduced tumors.

Although we showed that high B7-1–expressing B-16 melanoma was rejected in vivo (Fig. 2), Chen et al. demonstrated that B7-1–transduced B-16 cells were tumorigenic (22). The discrepancy between our data and those of Chen et al. can be explained by serveral possibilities. First, the level of B7-1 expression may be different between the tumors used in these two studies. It is possible that B7-1 expression was significantly higher in our B7-1–transduced B-16 tumor cells. We have shown in Fig. 2 that different levels of B7-1 expression in the tumor cells have different outcomes in the tumorigenicity. For example, B-16 melanoma cells with intermediate levels of B7-1 expression were shown to be tumorigenic. Second, the heterogeneity of high B7-1–expressing tumor cells within the whole tumor population injected may influence the outcome in tumor growth. We have shown that even small quantities of wild-type B-16 tumor cells mixed with high B7-1–expressing tumor cells can lead to tumor growth in mice (Fig. 3). Furthermore, the explanted tumor cells from mice injected with 100% high expressing B16 cells demonstrated decreased B7-1 expression in explanted tumor cells (Fig. 4). These data indicate that bystander high B7-1–expressing tumor cells do not help in the elimination of neighboring low or negative B7-1–expressing tumors. The small quantity of low or negative B7-1–expressing B16 tumor cells might eventually outgrow and form tumor nodules in the injected mice.

Certain B7-1–expressing MHC class II^+ tumors have been shown to be rejected through the function of $CD4^+$ T cells (14). In our study, NK cells and $CD8^+$ T cells, but not $CD4^+$ T cells, are critical in the in vivo rejection of high B7-1–expressing B-16 cells. This may be related to the fact that B16 tumor cells do not express MHC class II molecules (data not shown).

NK1.1 cells are found to be important in the in vivo rejection of high B7-1–expressing B16 tumor cells as demonstrated by our in vivo antibody depletion experiment (see Fig. 5). This is somewhat an unexpected result since most previous in vivo studies mainly emphasized the importance

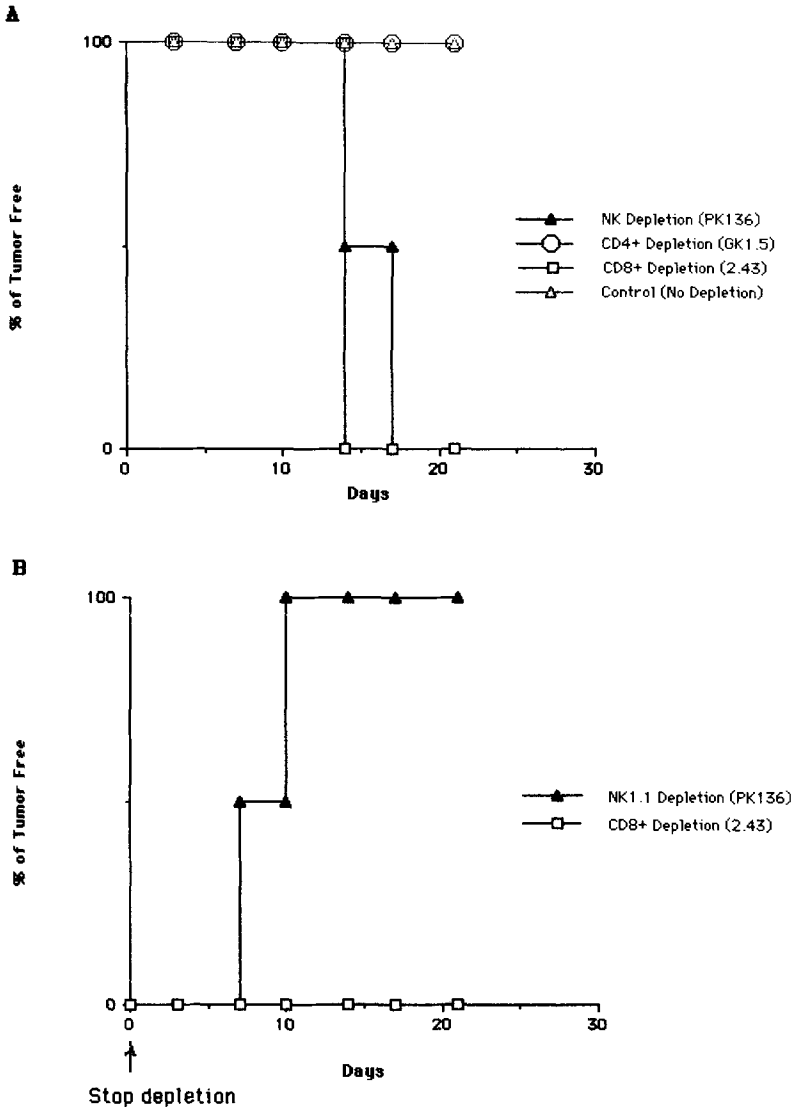

Figure 5. Effect of lymphocyte subset depletions on the tumor growth of B7-1^{hi}–expressing B-16. For each C57BL/6 mouse, 10^6 of B7-1^{hi}–expressing MC1 were injected subcutaneously. (*A*) Tumor growth with in vivo antibody depletions. Depletions were started 1 wk before tumor inoculation. mAb GK1.5 (18) was used for CD4$^+$ T cell depletion (*open triangles*), mAb 2.43 (19) was used for CD8$^+$ T cell depletion (*open squares*), and mAb PK136 (20) was used for NK1.1 cell depletion (*closed triangles*). Mice without lymphocyte subset depletions were used as control (*open circles*). Tumor growth was measured regularly. (*B*) Tumor growth after termination of in vivo antibody depletions. Termination of antibody depletions is indicated by an arrow on day 0 (day 21 after tumor injection). 1 wk after termination of depletion (day 28 after inoculation of tumor cells), mice that were once treated with NK1.1 depletion (*closed triangles*) began to show regression of tumors. In contrast, the tumor continued to grow in those mice that were once treated with CD8$^+$ T cell depletion (*open squares*). 10 mice were used per group, and each experiment was repeated at least once.

of CD8$^+$ (13, 15) and CD4$^+$ (14) T lymphocytes in the rejection of B7-1$^+$ tumors. Nevertheless, B16 tumors have low MHC class I expression, and NK cell sensitivity can be induced through loss of MHC class I molecules(for review see reference 23). NK cells may therefore be important in the killing of B16 tumor cells.

The B7-1 expression in the B16 tumors may play a role in NK cell activation. At least a subset of NK cells expresses CD28 (24, 25). NK cells have been shown to interact with B7-1 molecules on tumor cells in in vitro models (24). For example, YT2C2, a human NK leukemia cell line, expresses CD28 and can spontaneously lyse both murine and human cell lines expressing B7-1 in vitro (24). The participation of CD28/B7-1 interactions in NK-mediated cytotoxicity has been demonstrated by the correlation of target sensitivity with levels of B7-1 expression in target cells (24). The efficient lysis of B7-1–expressing target cells by NK cells, however, might require additional interactions of other "adhesion" molecules with their respective ligands (24). In addition, the CD28 costimulatory pathway has been reported to be important in the proliferation and cytokine production of NK cells (25).

The mechanism for NK cell–mediated lysis is not completely understood. NK cells can selectively kill transformed, virus- and intracellular bacteria–infected cells (for review see reference 26). NK cells are also able to mediate rejection of bone marrow allografts (27). The specific receptors used by NK cells for recognition and activation remain poorly defined, but they probably include both triggering and inhibitory molecules (28, 29). MHC class I expression by target cells inhibits lysis mediated by NK cells, often in an allele-specific fashion (30). It has been proposed that NK cell inhibitory receptors recognize complexes of class I molecules with cellular peptides that define self. Alteration of these self peptides could render these cells sensitive to NK lysis. The peptides that define "self" can be either specific (31) or diverse (32), depending on the systems employed.

The fact that NK cells can recognize and lyse tumor targets without restriction to the MHC has important implications in the design of immunotherapeutic strategies and vaccine development against tumors since a significant proportion of tumors has been found to express low or negative expression of MHC molecules. The loss of MHC class I antigens has been observed in a wide range of tumors, including breast (33), lung (34), ovary (35), skin (36), melanoma (37), cervix (38), colon, and rectum (39–44), to name a few. It will be important to better understand how NK cells recognize and lyse tumor targets to design better strategies for enhancing antitumor responses against low or negative MHC class I–expressing tumor cells.

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References

1. Hellstrom, K.E., and I. Hellstrom. 1991. Principles of tumor immunity: tumor antigen. *In* The Biologic Therapy of Cancer. V.T. De Vita, Jr., S. Hellman, and S.A. Rosenberg, editors. J.B. Lippincott Co., Philadelphia. pp. 35–52.
2. Melief, C.J. 1992. Tumor eradication by adoptive transfer of cytotoxic T lymphocytes. [Review]. *Adv. Cancer Res.* 58: 143–75.
3. Greenberg, P.D. 1991. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. [Review]. *Adv. Immunol.* 49:281–355.
4. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu. Rev. Immunol.* 7:445–480.
5. Jenkins, M.K., and J.G. Johnson. 1993. Molecules involved in T-cell costimulation. [Review]. *Curr. Opin. Immunol.* 5: 361–367.
6. Janeway, C.J., and K. Bottomly. 1994. Signals and signs for lymphocyte responses. [Review]. *Cell.* 76:275–285.
7. Schwartz, R.H. 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. [Review]. *Cell.* 71:1065–1068.
8. Freeman, G.J., G.S. Gray, C.D. Gimmi, D.B. Lombard, L.J. Zhou, M. White, J.D. Fingeroth, J.G. Gribben, and L.M. Nadler. 1991. Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. *J. Exp. Med.* 174:625–631.
9. Azuma, M., D. Ito, H. Yagita, K. Okumura, J.H. Phillips, L.L. Lanier, and C. Somoza. 1993. B70 antigen is a second ligand for CTLA-4 and CD28. *Nature (Lond.).* 366:76–79.
10. Freeman, G.J., F. Borriello, R.J. Hodes, H. Reiser, K.S. Hathcock, G. Laszlo, A.J. McKnight, J. Kim, L. Du, D.B. Lombard, et al. 1993. Uncovering of functional alternative CTLA-4 counter-receptor in B7-deficient mice [see comments]. *Science (Wash. DC).* 262:907–909.
11. Freeman, G.J., J.G. Gribben, V.A. Boussiotis, J.W. Ng, V.J. Restivo, L.A. Lombard, G.S. Gray, and L.M. Nadler. 1993. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation [see comments]. *Science (Wash. DC).* 262:909–911.
12. Linsley, P.S., W. Brady, M. Urnes, L.S. Grosmaire, N.K. Damle, and J.A. Ledbetter. 1991. CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* 174: 561–569.
13. Chen, L., S. Ashe, W.A. Brady, I. Hellstrom, K.E. Hellstrom, J.A. Ledbetter, P. McGowan, and P.S. Linsley. 1992. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell.* 71:1093–1102.
14. Baskar, S., R.S. Ostrand, N. Nabavi, L.M. Nadler, G.J. Freeman, and L.H. Glimcher. 1993. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules. *Proc. Natl. Acad. Sci. USA.* 90:5687–5690.
15. Townsend, S.E., and J.P. Allison. 1993. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells [see comments]. *Science (Wash. DC).* 259: 368–370.
16. Dranoff, G., E. Jaffee, A. Lazenby, P. Golumbek, H. Levitsky, K. Brose, V. Jackson, H. Hamada, D. Pardoll, and R.C. Mulligan. 1993. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA.* 90:3539–3543.
17. Levitsky, H.I., A. Lazenby, R.J. Hayashi, and D.M. Pardoll. 1994. In vivo priming of two distinct antitumor effector populations: the role of major histocompatibility complex class I expression. *J. Exp. Med.* 179:1215–1224.
18. Dialynas, D.P., Z.S. Quan, K.A. Wall, A. Pierres, J. Quintans, M.R. Loken, M. Pierres, and F.W. Fitch. 1983. Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. *J. Immunol.* 131: 2445–2451.
19. Sarmiento, M., A.L. Glasebrook, and F.W. Fitch. 1980. IgG

or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt 2 antigen block T cell-mediated cytolysis in the absence of complement. *J. Immunol.* 125:2665–2672.
20. Koo, G.C., F.J. Dumont, M. Tutt, J. Hackett, Jr., and V. Kumar. 1986. The NK-1.1(−) mouse: a model to study differentiation of murine NK cells. *J. Immunol.* 137:3742–3747.
21. Huang, A.Y., P. Golumbek, M. Ahmadzadeh, E. Jaffee, D. Pardoll, and H. Levitsky. 1994. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science (Wash. DC).* 264:961–965.
22. Chen, L., P. Mcgowan, S. Ashe, J. Johnston, Y. Li, I. Hellstrom, and K. Hellstrom. 1994. Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. *J. Exp. Med.* 179:523–532.
23. Ljunggren, H., and K. Karre. 1990. In search of the "missing self": MHC molecules and NK cell recognition. *Immunol. Today.* 11:237–244.
24. Azuma, M., M. Cayabyab, D. Buck, J.H. Phillips, and L.L. Lanier. 1992. Involvement of CD28 in MHC-unrestricted cytotoxicity mediated by a human natural killer leukemia cell line. *J. Immunol.* 149:1115–1123.
25. Nandi, D., J.A. Gross, and J.P. Allison. 1994. CD28-mediated costimulation is necessary for optimal proliferation of murine NK cells. *J. Immunol.* 152:3361–3369.
26. Trinchieri, G. 1989. Biology of natural killer cells. [Review]. *Adv. Immunol.* 47:187–376.
27. Yu, Y.L.L., V. Kumar, and M. Bennett. 1992. Murine natural killer cells and marrow graft rejection. *Annu. Rev. Immunol.* 10:189–213.
28. Correa, I., L. Corral, and D.H. Raulet. 1994. Multiple natural killer cell-activating signals are inhibited by major histocompatibility complex class I expression in target cells. *Eur. J. Immunol.* 24:1323–1331.
29. Storkus, W.J., and J.R. Dawson. 1991. Target structures involved in natural killing (NK): characteristics, distribution, and candidate molecules. [Review]. *Crit. Rev. Immunol.* 10: 393–416.
30. Sentman, C.L., J. Hackett, Jr., V. Kumar, and M. Bennett. 1989. Identification of a subset of murine NK cells that mediates rejection of Hh-1d but not Hh-1b bone marrow grafts. *J. Exp. Med.* 170:191–202.
31. Malnati, M.S., M. Peruzzi, K.C. Parker, W.E. Biddison, E. Ciccone, A. Moretta, and E.O. Long. 1995. Peptide specificity in the recognition of MHC class I by natural killer cell clones. *Science (Wash. DC).* 267:1016–1018.
32. Correa, I., and D.H. Raulet. 1995. Binding of diverse peptides to MHC class I molecules inhibits target cell lysis by activated natural killer cells. *Immunity.* 2:61–71.
33. Fleming, K.A., A. McMichael, J.A. Morton, J. Woods, and J.O. McGee. 1981. Distribution of HLA class 1 antigens in normal human tissue and in mammary cancer. *J. Clin. Pathol.* 34:779–784.
34. Doyle, A., W.J. Martin, K. Funa, A. Gazdar, D. Carney, S.E. Martin, I. Linnoila, F. Cuttitta, J. Mulshine, P. Bunn, et al. 1985. Markedly decreased expression of class I histocompatibility antigens, protein, and mRNA in human small-cell lung cancer. *J. Exp. Med.* 161:1135-1151.
35. Kabawat, S.E., R.C. Bast, Jr., W.R. Welch, R.C. Knapp, and A.K. Bhan. 1983. Expression of major histocompatibility antigens and nature of inflammatory cellular infiltrate in ovarian neoplasms. *Int. J. Cancer.* 32:547–554.
36. Natali, P.G., M. Viora, M.R. Nicotra, P. Giacomini, A. Bigotti, and S. Ferrone. 1983. Antigenic heterogeneity of skin tumors of nonmelanocyte origin: analysis with monoclonal antibodies to tumor-associated antigens and to histocompatibility antigens. *J. Natl. Cancer Inst.* 71:439–447.
37. Natali, P.G., A. Bigotti, R. Cavaliere, M.R. Nicotra, and S. Ferrone. 1984. Phenotyping of lesions of melanocyte origin with monoclonal antibodies to melanoma-associated antigens and to HLA antigens. *J. Natl. Cancer Inst.* 73:13–24.
38. Connor, M., and P. Stern. 1990. Loss of MHC class-I expression in cervical carcinomas. *Int. J. Cancer.* 46:1029–1034.
39. Csiba, A., H.L. Whitwell, and M. Moore. 1984. Distribution of histocompatibility and leucocyte differentiation antigens in normal human colon and in benign and malignant colonic neoplasms. *Br. J. Cancer.* 50:699–709.
40. Lopez-Nevot, M.A., E. Garcia, C. Romero, M.R. Oliva, S. Serrano, and F. Garrido. 1988. Phenotypic and genetic analysis of HLA class I and HLA-DR antigen expression on human melanomas. *Exp. Clin. Immunogenet.* 5:203-212.
41. Lopez-Nevot, M.A., F. Esteban, A. Ferron, J. Gutierrez, M.R. Oliva, C. Romero, C. Huelin, F. Ruiz-Cabello, and F. Garrido. 1989. HLA class I gene expression on human primary tumours and autologous metastases: demonstration of selective losses of HLA antigens on colorectal, gastric and laryngeal carcinomas. *Br. J. Cancer.* 59:221-226.
42. Momburg, F., T. Degener, E. Bacchus, G. Moldenhauer, G.J. Hammerling, and P. Moller. 1986. Loss of HLA-A,B,C and de novo expression of HLA-D in colorectal cancer. *Int. J. Cancer.* 37:179–184.
43. Momburg, F., A. Ziegler, J. Harpprecht, P. Moller, G. Moldenhauer, and G.J. Hammerling. 1989. Selective loss of HLA-A or HLA-B antigen expression in colon carcinoma. *J. Immunol.* 142:352–358.
44. van den Ingh, H.F., D.J. Ruiter, G. Griffioen, G.N. van Muijen, and S. Ferrone. 1987. HLA antigens in colorectal tumours—low expression of HLA class I antigens in mucinous colorectal carcinomas. *Br. J. Cancer.* 55:125–130.