Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules

(CD28/tumor immunity)

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ABSTRACT   The inability of the autologous host to reject resident tumor cells is frequently the result of inadequate generation of tumor-specific T cells. Specific activation of T cells occurs after delivery of two signals by the antigen-presenting cell. The first signal is antigen-specific and is the engagement of the T-cell antigen receptor by a specific major histocompatibility complex antigen–peptide complex. For some T cells, the second or costimulatory signal is the interaction of the T-cell CD28 receptor with the B7 activation molecule of the antigen-presenting cell. In the present study, we demonstrate that mouse sarcoma cells genetically engineered to provide both T-cell activation signals stimulate potent tumor-specific CD4+ T cells that cause rejection of both engineered and wild-type neoplastic cells. Two other recent studies have also demonstrated that costimulation via B7 can improve tumor immunity. However, our study differs from these reports by two important observations. (i) One of these studies utilized mouse tumor cells expressing xenogeneic viral antigens, and hence, the results are not applicable to wild-type resident tumors. Our study, however, demonstrates that coexpression of B7 by major histocompatibility complex class II+ tumor cells induces immunity in the autologous host that is specific for naturally occurring tumor antigens of poorly immunogenic tumors. (ii) In both earlier studies, only CD8+ T cells were activated after coexpression of B7, whereas in the present report, tumor-specific CD4+ T cells are generated. This report therefore illustrates the role of the B7 activation molecule in stimulating potent tumor-specific CD4+ T cells that mediate rejection of wild-type tumors and provides a theoretical basis for immunotherapy of established tumors.

Rejection of a tumor by the autologous host is often mediated by tumor-specific T lymphocytes. Recent studies from a number of laboratories (1–3) suggest that the inability of the host to reject a resident tumor may be due to the insufficient generation of tumor-specific T helper lymphocytes. CD4+ T helper cells are specifically activated when they receive two signals delivered by an appropriate antigen-presenting cell (APC) (4). The first signal is the engagement of the antigen-specific T-cell receptor by the major histocompatibility complex (MHC) class II antigen–peptide complex. The second or costimulatory signal can vary from system to system, but for at least some lymphocytes, it is the binding of the B7 molecule to its cognate receptor, CD28, on the responding T cell (5–8). In this report we show that malignant tumor cells can be highly effective immunogens in the autologous host if they are engineered to present tumor antigen and deliver the

B7 coactivation signal. Immunization with such engineered tumor cells generates potent tumor-specific CD4+ T cells that facilitate rejection and confer immunologic memory to high-dose challenges of wild-type neoplastic cells. These results demonstrate the critical role of the B7 costimulatory pathway in stimulating tumor-specific CD4+ T cells and provide an attractive strategy for enhancing tumor immunity.

MATERIALS AND METHODS

Cells. SaI tumor cells were maintained as described (1).

Antibodies. The monoclonal antibody (mAb) 10-3.6, specific for I-A^k (9), was prepared and used as described (1). The B7-specific mAb 1G10 is a rat IgG2a mAb and was used as described (10). mAbs specific for CD4+ [GK1.5 (11)] and CD8+ [2.43 (12)] were used as ascites fluid.

Transfections. Mouse SaI sarcoma cells were transfected as described (1) with wild-type Aa^k and Ab^k MHC class II cDNAs, Aa^k and Ab^k cDNAs truncated for their C-terminal 12 and 10 amino acids, respectively (13), and/or B7 gene (14). Class II transfectants were cotransfected with pSV2neo plasmid and selected for resistance to G418 (400 μg/ml). B7 transfectants were cotransfected with pSV2hph plasmid and selected for hygromycin-resistance (400 μg/ml). All transfectants were cloned twice by limiting dilution, except SaI/B7 transfectants, which were uncloned, and maintained in drug. Double transfectants were maintained in G418 plus hygromycin. The numbers after each transfectant are the clone designation.

Immunofluorescence. Indirect immunofluorescence was performed as described (1), and samples were analyzed on an Epics C flow cytometer.

Tumor Challenges. For primary tumor challenges, autologous A/J mice were challenged i.p. with the indicated number of tumor cells. Inoculated mice were checked three times per week for tumor growth. Mean survival times of mice dying from their tumor ranged from 13 to 28 days after inoculation. Mice were considered to have died from their tumor if they contained a large volume of ascites fluid and tumor cells (≥5 ml) at the time of death. Mice were considered tumor-resistant if they were tumor-free for at least 60 days after tumor challenge (range, 60–120 days). Tumor cells were monitored by indirect immunofluorescence for I-A^k and B7 expression prior to tumor-cell inoculation. For the experiments of Table 2, autologous A/J mice were immunized i.p. with a single inoculum of the indicated number of live tumor cells and challenged i.p. with the indicated number of wild-

Abbreviations: MHC, major histocompatibility complex; APC, antigen-presenting cell; mAb, monoclonal antibody.
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type SaI cells 42 days after immunization. Mice were evaluated for tumor resistance or susceptibility using the same criteria as for primary tumor challenge.

In vivo **T-Cell Depletions.** A/J mice were depleted for CD4$^+$ or CD8$^+$ T cells by i.p. inoculation with 100 μl of ascites fluid of mAb GK1.5 (CD4$^+$ specific; ref. 11) or mAb 2.43 (CD8$^+$ specific; ref. 12) on days −6, −3, and −1 prior to tumor challenge, and every third day after tumor challenge as described (15) until the mice died or day 28, whichever came first. Presence or absence of tumor was assessed up to day 28. Previous studies have established that A/J mice with large tumors at day 28 after injection will progress to death. This time point was, therefore, chosen to assess tumor susceptibility for the *in vivo* depletion experiments. One mouse per group was sacrificed on day 28, and its spleen was assayed by immunofluorescence to ascertain depletion of the relevant T-cell population.

RESULTS

Coexpression of B7 Compensates for the Absence of the MHC Class II Cytoplasmic Domain and Restores Immunogenicity.

The mouse SaI sarcoma is an ascites-adapted class I$^+$ class II$^-$ tumor of A/J (*H-2K^{k}A^{k}D^d*) mice. The wild-type tumor is lethal in autologous A/J mice when administered i.p. SaI cells transfected with, and expressing, syngeneic MHC class II genes (*Aa^k* and *Ab^k* genes; SaI/A^k cells) are immunologically rejected by the autologous host, and immunization with live SaI/A^k cells protects mice against subsequent challenges with wild-type class II$^-$ SaI cells (1). Adoptive transfer (16) and lymphocyte depletion studies (E. Lamoussé-Smith and S.O.-R., unpublished data) demonstrate that SaI and SaI/A^k rejection is dependent on CD4$^+$ lymphocytes. SaI cells expressing class II molecules with truncated cytoplasmic domains (SaI/A^{k}tr cells), however, are as lethal as wild-type class II$^-$ SaI cells, suggesting that the cytoplasmic region of the class II heterodimer is required to induce protective immunity (17).

It has recently been demonstrated that up-regulation of the B7 activation molecule on the APC is triggered by intracellular signals transmitted by the cytoplasmic domain of the class II heterodimer, after presentation of antigen to CD4$^+$ T helper cells (10). Inasmuch as B7 expression is normally up-regulated *in vivo* on SaI cells expressing full-length class II molecules (S.B. and S.O.-R., unpublished data), we have speculated that SaI/A^{k}tr cells do not stimulate protective immunity because they do not transmit a costimulatory signal.

To test whether B7 expression can compensate for the absence of the class II cytoplasmic domain, SaI/A^{k}tr cells were supertransfected with a plasmid containing a cDNA encoding murine B7 under the control of the cytomegalovirus promoter and screened for I-A^k and B7 expression by indirect immunofluorescence. Wild-type SaI cells do not express either I-A^k or B7 (Fig. 1 *a* and *b*), whereas SaI cells transfected with *Aa^k* and *Ab^k* genes (SaI/A^k cells) or truncated *Aa^k* and *Ab^k* genes (SaI/A^{k}tr cells) express I-A^k (Fig. 1 *d* and *f*) and do not express B7 (Fig. 1 *c* and *e*). SaI cells transfected with truncated class II genes plus the B7 gene (SaI/A^{k}tr/B7 cells) express I-A^k and B7 molecules (Fig. 1 *g* and *h*). All cells express uniform levels of MHC class I molecules (K^k and D^d) comparable to the level of I-A^k in Fig. 1*h* (data not shown).

Antigen-presenting activity of the transfectants was tested by determining their immunogenicity and lethality in autologous A/J mice. As shown in Table 1, wild-type SaI cells administered i.p. at doses as low as 10^4 cells are lethal in 88–100% of mice inoculated within 13–28 days after challenge, whereas 100 times as many SaI/A^k cells are uniformly rejected. Challenges with similar quantities of SaI/A^{k}tr cells are also lethal; however, SaI/A^{k}tr cells that coexpress B7
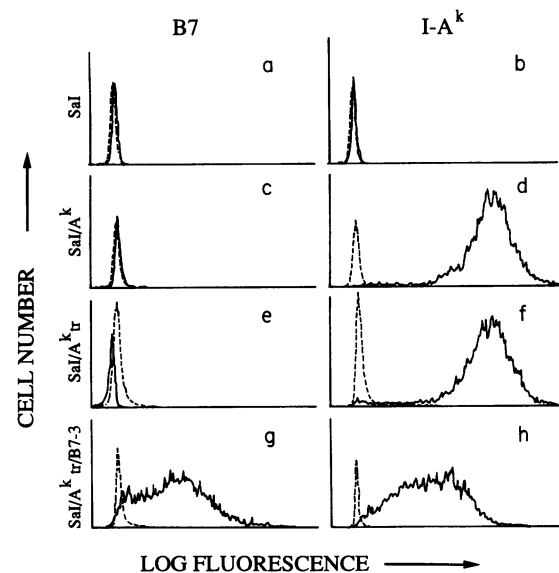

FIG. 1. SaI tumor cells transfected with *I-A^k* and B7 genes express these molecules at the cell surface. SaI/A^k, SaI cells transfected with wild-type *Aa^k* and *Ab^k* genes, clone 19.6.4; SaI/A^{k}tr, SaI cells transfected with truncated *Aa^k* and *Ab^k* genes, clone 6.11.8; SaI/A^{k}tr/B7, SaI cells transfected with truncated *Aa^k* and *Ab^k* genes and supertransfected with the B7 gene. All SaI/A^{k}tr/B7 clones tested consistently express lower levels of MHC class II antigen than SaI/A^{k}tr or SaI/A^k cells. Abscissa represents three orders of magnitude of fluorescence intensity. Dotted lines represent control immunofluorescent staining by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (*b, d, f,* and *h*) or FITC-conjugated goat anti-rat immunoglobulin (*a, c, e,* and *g*); solid lines represent staining by I-A^k-specific mAb 10-3.6 (9) plus FITC-conjugated goat anti-mouse immunoglobulin (*b, d, f,* and *h*) or B7-specific mAb 1G10 (10) plus FITC-conjugated goat anti-rat immunoglobulin (*a, c, e,* and *g*).

(SaI/A^{k}tr/B7 clones -1 and -3) are uniformly rejected. A/J mice challenged with SaI/A^{k}tr cells transfected with the B7 construct, but not expressing detectable amounts of B7 antigen (SaI/A^{k}tr/hph cells), are as lethal as SaI/A^{k}tr cells, demonstrating that reversal of the malignant phenotype in SaI/A^{k}tr/B7 cells is due to expression of B7. SaI cells transfected with the B7 gene and not coexpressing truncated class II molecules (SaI/B7 cells, uncloned) are also as lethal as wild-type SaI cells, indicating that B7 expression without truncated class II molecules does not stimulate immunity. To

Table 1. Tumorigenicity of B7 and MHC class II-transfected SaI tumor cells

Challenge tumor	Expression		Tumor dose, no. of cells	Mice dead/mice tested, no./no.
	I-A^k	B7		
SaI	−	−	1 × 10^6	9/10
	−	−	1 × 10^5	8/10
	−	−	1 × 10^4	7/8
SaI/A^k 19.6.4	A^k	−	1 × 10^6	0/12
	A^k	−	5 × 10^5	0/5
	A^k	−	1 × 10^5	0/5
SaI/A^{k}tr 6.11.8	A^{k}tr	−	1 × 10^6	12/12
	A^{k}tr	−	5 × 10^5	5/5
	A^{k}tr	−	1 × 10^5	5/10
SaI/A^{k}tr/B7-1	A^{k}tr	B7	1 × 10^6	0/4
SaI/A^{k}tr/B7-3	A^{k}tr	B7	1 × 10^6	0/5
	A^{k}tr	B7	4 × 10^5	0/5
	A^{k}tr	B7	1 × 10^5	0/5
SaI/A^{k}tr/hph	A^{k}tr	−	1 × 10^6	5/5
SaI/B7	−	B7	1 × 10^6	5/5

ascertain that rejection of SaI/A^k and SaI/A^{k}tr/B7 cells is immunologically mediated, sublethally irradiated (900 rads; 1 rad = 0.01 Gy) A/J mice were challenged i.p. with these cells. In all cases, irradiated mice died from the tumor. We conclude that immunogenicity and host rejection of the MHC class II$^+$ tumor cells are dependent on an intact class II molecule and that coexpression of B7 can bypass the requirement for the class II intracellular domain.

Immunization with B7-Transfected Sarcoma Cells Protects Against Later Challenges of Wild-Type B7$^-$ Sarcoma. Activation of at least some T cells is thought to be dependent on coexpression of B7. However, once the T cells are activated, B7 expression is not required on the target cell for recognition by effector T cells. We have therefore tested the ability of three SaI/A^{k}tr/B7 clones (B7-3, B7-1, and B7-2B5.E2) to immunize A/J mice against subsequent challenges of wild-type class II$^-$ B7$^-$ SaI cells (Table 2). A/J mice were immunized with live SaI/A^{k}tr/B7 transfectants and 42 days later challenged with wild-type SaI tumor cells. Ninety-seven percent of mice immunized with the SaI/A^{k}tr/B7 transfectants were immune to ≥10^6 wild-type B7$^-$ class II$^-$ SaI cells, an immunity that is comparable to that induced by immunization with SaI cells expressing full-length class II molecules. SaI/A^{k}tr/B7 cells, therefore, stimulate a potent response with long-term immunological memory against high-dose challenges of malignant tumor cells. B7 expression is, therefore, critical for the stimulation of SaI-specific effector cells; however, its expression is not needed on the tumor targets once the appropriate effector T-cell populations have been generated.

Immunization with B7-Transfected Tumor Cells Stimulates Tumor-Specific CD4$^+$ Lymphocytes. To ascertain that B7 is functioning through a T-cell pathway in tumor rejection, we have *in vivo*-depleted A/J mice for CD4$^+$ or CD8$^+$ T cells and challenged them i.p. with SaI/A^k or SaI/A^{k}tr/B7 cells. As shown in Table 3, *in vivo* depletion of CD4$^+$ T cells results in host susceptibility to both SaI/A^k and SaI/A^{k}tr/B7 tumors, indicating that CD4$^+$ T cells are critical for tumor rejection, whereas depletion of CD8$^+$ T cells does not affect SaI/A^{k}tr/B7 tumor rejection. Although immunofluorescence analysis of splenocytes of CD8$^+$-depleted mice demonstrates the absence of CD8$^+$ T cells, it is possible that the depleted mice contain small quantities of CD8$^+$ cells that are below our level of detection. These data therefore demonstrate that CD4$^+$ T cells are required for tumor rejection but do not eliminate a possible corequirement for CD8$^+$ T cells.

Previous adoptive transfer experiments (16) have demonstrated that both CD4$^+$ and CD8$^+$ T cells are required for rejection of class II$^-$ wild-type SaI cells. Inasmuch as rejec-

Table 3. Tumor susceptibility of A/J mice *in vivo*-depleted for CD4$^+$ or CD8$^+$ T cells

Tumor challenge	Host T-cell depletion	No. mice with tumor/ total no. mice challenged
SaI/A^k	CD4$^+$	3/5
SaI/A^{k}tr/B7-3	CD4$^+$	5/5
	CD8$^+$	0/5

tion of SaI/A^k and SaI/A^{k}tr/B7 cells appears to require only CD4$^+$ T cells, it is likely that immunization with class II$^+$ transfectants stimulates both CD4$^+$ and CD8$^+$ effector T cells; however, only the CD8$^+$ effectors are required for rejection of class I$^+$II$^-$ tumor targets. Costimulation by B7, therefore, enhances immunity by stimulating tumor-specific CD4$^+$ helper and cytotoxic lymphocytes.

DISCUSSION

In other recent studies, we have shown (18) that SaI/A^k cells supertransfected with the class II-associated invariant chain gene (Ii) are as malignant as wild-type SaI cells, indicating that class II$^+$ tumor cells that coexpress Ii are unable to stimulate tumor-specific immunity. Inasmuch as Ii is thought to inhibit the presentation of endogenously synthesized peptides by class II molecules (19–26), these data suggest that the increased immunogenicity of SaI/A^k cells is due to the presentation of endogenously synthesized tumor peptides. Collectively, these data are consistent with the hypothesis that Ii$^-$ SaI/A^k cells stimulate potent tumor-specific immunity because their class II molecules directly present endogenously synthesized tumor peptides to CD4$^+$ T cells, thereby improving the generation of tumor-specific T helper cells. The ability of the class II$^+$ tumor cells to directly present tumor peptides to CD4$^+$ T helper cells bypasses the need for third-party APCs and probably improves tumor immunogenicity because soluble tumor antigen (in the form of tumor-cell debris or secreted protein) may not be available for uptake by professional APCs.

Inasmuch as rejection of the SaI sarcoma by autologous A/J mice is T-cell-mediated, these results support the two-signal model for T-cell activation in primary immune responses. Previous studies have established the requirement for a second signal for activation of T cells *in vitro* (5–8); however, the present results document the requirement for both first and second signals for effective T-cell activation within the complex *in vivo* setting of autologous tumor rejection.

The requirement for a costimulatory signal for generation of effective tumor-specific immunity raises the question of whether inadequate anti-tumor responses are due to insufficient generation of a first or second signal. Indeed, in the absence of costimulation, tumor-specific T cells may be anergized, leading to tolerance (4). This scenario may occur in malignant disease if tumor-cell debris is not present or if tumor antigens are not secreted, and hence, tumor peptides are not available for uptake by APCs that constitutively express costimulatory molecules such as the B7 activation antigen.

Although SaI is a weakly immunogenic tumor, it can induce effective tumor-specific immunity if, by transfection, it expresses the appropriate antigen-presenting elements (i.e., MHC class II molecules) and delivers the required signals (e.g., B7) to responding T cells. The inability of the autologous host to respond to wild-type tumor cells is, therefore, probably not due to lack of expression of tumor peptides but rather to inadequate presentation of these peptides and/or to delivery of the required additional activation signals.

Table 2. Autologous A/J mice immunized with SaI/A^{k}tr/B7 cells are immune to challenges of wild-type SaI tumor

Immunization	No. of immunizing cells	SaI challenge dose, no. of cells	Mice dead/ mice tested, no./no.
None	—	1 × 10^6	5/5
SaI/A^k 19.6.4	1 × 10^5 or 10^6	1 × 10^6	0/5
	1 × 10^6	6 × 10^6	0/5
SaI/A^{k}tr/B7-3	1 × 10^6	6 × 10^6	0/5
	1 × 10^6	1 × 10^6	0/5
	4 × 10^5	1 × 10^6	0/5
	1 × 10^5	5 × 10^6	0/5
SaI/A^{k}tr/B7-1	5 × 10^5	3 × 10^6	0/3
	2 × 10^5	1 × 10^6	0/2
	5 × 10^4	5 × 10^6	0/3
SaI/A^{k}tr/B7-2B5.E2	1 × 10^5	2 × 10^6	0/2
	5 × 10^4	2 × 10^6	1/7

Two other reports (27, 28) have also demonstrated the efficacy of B7 expression for improving tumor-specific immunity; however, two important differences distinguish the present report from these studies. In both of the previous studies, the K1735 mouse melanoma was transfected with the B7 gene. Interestingly, Chen *et al.* (27) cotransfected with the E7 viral gene from human papillomavirus, and the resulting immunity was specific for and dependent on expression of the E7 gene product. Inasmuch as E7$^-$ melanoma cells were not targets for B7-stimulated effectors, this study suggested that constitutive B7 expression would not be applicable as immunotherapy for wild-type established tumors. In the Townsend and Allison study (28), however, using the same K1735 tumor, coexpression of a viral antigen was not required for immunity. Likewise, in our study, expression of a xenogeneic tumor antigen is not required, and immunity appears to be directed against endogenously encoded murine tumor molecules. Hence, our studies support the contention that coexpression of B7 can stimulate potent immunity to natural tumor antigens and, therefore, provide a strong experimental basis for stimulating immunity to spontaneous resident malignancies.

In the present report, we demonstrate that B7-transfected sarcoma cells stimulate potent tumor-specific CD4$^+$ effector cells, whereas in the studies of Chen *et al.* (27) and Townsend and Allison (28), immunization with B7-transfected melanoma cells induced CD8$^+$ effectors. This difference in effector population is probably the result of the presentation of tumor peptide by different MHC gene products. In the K1735 melanoma system, the tumor antigen is most likely presented by MHC class I molecules, whereas in our sarcoma system tumor peptide is presented by MHC class II molecules. Collectively, the three studies demonstrate that under the appropriate conditions, coexpression of B7 can optimize stimulation of both CD4$^+$ and CD8$^+$ T cells, thereby enhancing the tumor-specific immune response in both T-cell compartments.

In the experimental system described in this report, constitutive expression of B7 appears to provide the costimulatory signal for T-cell activation in the absence of the MHC class II cytoplasmic domain. Aside from being a formal demonstration of the role of the class II cytoplasmic domain in second signal induction, this result provides an experimental framework for improving tumor-specific immunity. Our previous approach for improving tumor-specific responses has been to constitutively express syngeneic MHC class II molecules in tumor cells (1) and rely on the transient induction of costimulatory signals during the immunization process. However, a wider repertoire of tumor-specific T cells may be activated, resulting in a more potent primary response, if B7 is stably expressed by the class II$^+$ tumor. Tumor cells stably coexpressing B7 and syngeneic MHC class II molecules may, therefore, be very useful immunogens for protecting against subsequent metastatic disease and/or for rescuing individuals carrying established tumors.

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