Local Costimulation Reinvigorates Tumor-Specific Cytolytic T Lymphocytes for Experimental Therapy in Mice with Large Tumor Burdens[1]

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Cytotoxic T cells recognize tumor Ags and destroy cancer cells in vitro. Adoptive transfer studies with transgenic T cells specific for tumor Ags have demonstrated that CTL are effective only in mice with small tumor burdens and thus appear to have limited potential in cancer immunotherapy. Here we used transgenic mice that express the TCR specific for an unmutated tumor Ag P1A and multiple lineages of P1A-expressing tumors to address this critical issue. We found that local costimulation, either by expression of B7-1 on the tumor cells or by local administration of anti-CD28 mAb 37N, reinvigorated the function of CTL specific for the tumor Ag, as it substantially increased the efficacy of CTL therapy for mice with large tumor burdens. Our study suggests that CTL-based immunotherapy can be manipulated to deal with large tumors. ***The Journal of Immunology*****, 2001, 167: 3936–3943.**

A largely unresolved issue is why immune surveillance against cancer often fails. It has been suggested that tumor Ags are not efficiently recognized by T cells, because of either their confinement to the nonhemopoietic tissues (1, 2) or the context of tumor Ag presentation (3) and/or the tumor-mediated immunosuppression (4, 5). Although abundant evidence has been presented for each of the mechanisms in a variety of cancer models, recent analysis has revealed substantial expansion of cancer-specific T cells in many human cancer patients (6–8), which demonstrates that tumor Ags are perhaps often recognized by the host T cells. Although protection of these T cells was suggested in one study (6), in the majority of the cases, their clinical benefit was either absent or obscure. In experimental animals, we found that transgenic T cells specific for an unmutated tumor Ag were relatively inefficient in elimination of a tumor that expressed the Ag (9). Although T cells specific for a mutated tumor Ag were reported to be more efficient in dealing with early tumors, established tumors were resistant to high numbers of tumor-specific CTL (10). Because Ags in the tumors that originate from nonhemopoietic tissues may not be presented to the naive T cells until the tumors have been established, the inability of tumor-specific T cells to reject large tumors may also explain the poor immune surveillance of cancer. As such, one of the most difficult challenges in tumor immunotherapy is to enhance T cell effector function against large established tumors.

Identification of B7-1 and B7-2 as the prototypic costimulatory molecules has led to novel approaches to tumor immunotherapy (11–15). Although expression of these molecules on tumors often leads to the rejection of tumor cells by CD8 T cells (11, 13, 14), the mechanism for the effect is less clear. Based on the fact that costimulation promotes T cell clonal expansion, it has been suggested that B7-1 and B7-2 promote tumor rejection by enhancing the induction of antitumor T cell response (3). However, because the induction of CTL response can be mediated by host APC (3, 16–18), it is unlikely that expression of B7-1 and B7-2 on the tumors would be essential for clonal expansion of tumor-specific T cells. In this regard, we and others have suggested that B7-1 expressed on tumor cells promotes the effector function of CTL, including both autoreactive and tumor-reactive CTL (14, 19–21). A critical role for B7-1 at the effector phase may provide an opportunity to reinvigorate antitumor CTL. In this study, we used an adoptive transfer model to analyze the function of B7-1 on the tumor cells at the effector phase. Our results demonstrate that tumor-specific CTL preferentially eliminated B7-1$^+$ tumor cells even when the B7-1$^+$ and B7-1$^-$ were administered as a mixture and formed microchimerism in vivo. Remarkably, either expression of B7-1 on the tumor cells or local administration of a mAb specific for CD28 substantially increased the susceptibility of large tumors to tumor-specific T cells and led to a substantial clinical benefit to the host.

Materials and Methods

Experimental animals

Transgenic mice expressing TCR specific for tumor Ag P1A35-43:L^d complex have been described (9). The TCR transgenes were backcrossed with BALB/cByJ for at least seven generations before they were used for this study. BALB/c mice with the targeted mutation of the *RAG-2* gene were purchased from Taconic Farms (Germantown, NY).

Cell lines

Plasmacytoma J558 (BALB/c origin) transfected with either vector alone (J558-Neo) or wild-type B7-1 (J558-B7) has been described (14). B7$^+$ and B7$^-$ P815 (DBA/2 origin) and Meth A (BALB/c origin) tumor cells were provided by Dr. L. Chen (Mayo Clinic, Rochester, MN) and have been described (22).

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Abs and flow cytometry

Ascites of anti-CD28 mAb 37N (23) and anti-CTLA4 mAb 4F10 (24) were produced using hybridomas kindly provided by Drs. J. P. Allison (University of California, Berkeley, CA) and J. A. Bluestone (University of Chicago, Chicago, IL), respectively. Cell surface expression of B7-1 or L^d was detected using biotinylated anti-B7-1 mAb 10.16A or anti-L^d mAb HB-27 (BD PharMingen, San Diego, CA); cell surface-bound mAb was detected using PE-streptavidin. Abs against cell surface molecules such as Vα8 (PE), CD8 (CyChrome), CD62L (PE), CD43 (FITC, 1B11), and CD44 (FITC) were purchased from BD PharMingen.

Adoptive transfer of purified transgenic T cells

Pools of spleen and lymph node cells from the P1CTL-transgenic mice were incubated with a mixture of mAbs (anti-CD4 mAb GK1.5, anti-FcR mAb 2.4G2, anti-CD11c mAb N418); after removal of unbound mAbs, the cells were incubated with anti-Ig-coated magnetic beads. The Ab-coated cells were removed by a magnet. The unbound cells consisted of >90% CD8 T cells, with no detectable CD4 T cells. The purified T cells were adoptively transferred into RAG-$2^{-/-}$ mice that either had established tumors or had received tumor cells at the same day of adoptive transfer. In some experiments, the P1CTL were activated for 4 days with their cognate peptide ($P1A_{35-43}$, 0.1 μg/ml) in vitro before adoptive transfer.

Tumorigenicity assay

Given numbers of tumor cells were injected in the flanks of mice as described (14). The tumor size and incidence were determined by physical examination every other day after tumor inoculation.

Immunohistochemistry with anti-B7-1 mAb

Frozen sections of tumors were fixed with acetone and incubated with anti-B7-1 mAb 10.16A hybridoma (25) supernatants. After extensive washes, the anti-B7-1 mAb was detected by biotinylated goat anti-hamster Abs followed by HPR-conjugated streptavidin.

RNase protection assay

Total RNAs from spleens or tumor-infiltrating cells were isolated with Trizol reagent (Life Technologies, Gaithersburg, MD). The concentration of RNA in each sample was assessed spectrophotometrically. The multiprobe RNase protection assay kit (RiboQuant; BD PharMingen) was used, with the assay performed according to the manufacturer's protocol. Briefly, a set of ^{32}P-labeled RNA probes synthesized from DNA templates using T7 polymerase was hybridized with 5 μg total RNA, after which free probes and other single-stranded RNA were digested with RNases. The remaining RNase-protected probes were purified and then resolved on denaturing polyacrylamide gels. The following template sets for murine apoptosis and cytokines were used in the present study: $mAPO_2$: bcl-W, bfl-1, bcl-X, bak, bax, bcl-2, bad, L32, and GAPDH; mCK-3b: TNF-β, LTβ, TNF-α, IL-6, IFN-γ, IFN-β, TGF-β1, TGF-β2, TGF-β3, macrophage migration inhibiting factor (MIF), L32, and GAPDH.

Cytotoxicity assay

As the effectors, we used ex vivo spleen cells after a short term (12 h) reactivation in vitro with the P1A peptide (0.1 μg/ml). Data were presented as percent specific lysis. P338D1 cells, pulsed with tumor antigenic peptide P1A or a control L^d-binding viral peptide, were used as the targets.

Results

P1CTL preferentially eliminate multiple lineages of B7-1$^+$ tumor cells in vivo

To test whether P1CTL preferentially reject B7-1$^+$ tumor cells, we injected J558-B7 and J558-Neo tumor cells at separate flanks of the same RAG-$2^{-/-}$ mice. Some of the tumor-bearing mice were then adoptively transferred with purified CD8 T cells from P1CTL transgenic mice. As shown in Fig. 1, *top*, J558-Neo and J558-B7 grew at comparable rates in RAG-$2^{-/-}$ mice that received no T cells. In mice that received P1CTL, J558-B7 tumors either failed to develop (Fig. 1*A*) or developed in one of the mice and rapidly rejected (Fig. 1*B*). In contrast, J558-Neo tumors grew progressively even when large numbers of P1CTL were transferred, although the tumor growth was delayed by 1 or 2 wk, and the mice survived up to 1 mo longer than did the groups that received no T cells. Thus, P1CTL preferentially rejected the B7-1$^+$ tumor cells. The ability of P1CTL to reject the B7-1$^+$ tumors and to retard the development of the J558-Neo tumors demonstrates that CD8 T cells specific for a single peptide can reject tumors without participation of any other Ag-specific lymphocytes.

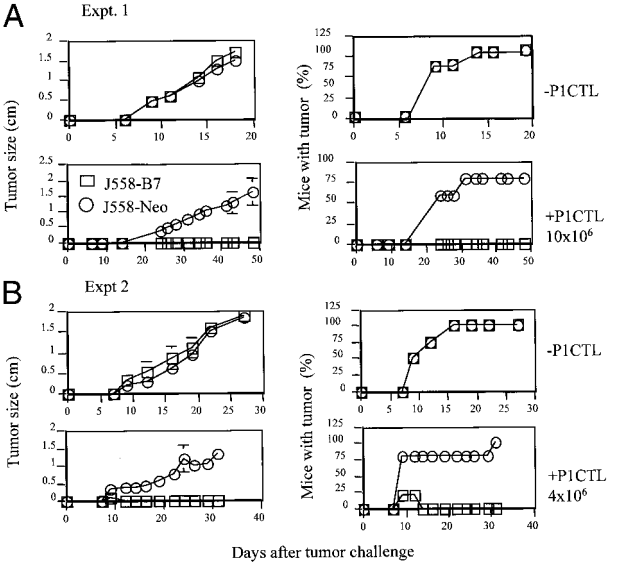

FIGURE 1. B7-dependent rejection of tumor. J558-B7 or J558-neo tumor cells (5×10^6) were injected into the same mouse in separate flanks. These mice were either left untreated or injected i.v. with 10×10^6 (*A*) or 4×10^6 (*B*) purified CD8$^+$ P1CTL-transgenic T cells at the same time. Tumor growth kinetics (*left column*) and tumor incidence (*right column*) were monitored after tumor inoculation.

We analyzed the frequency and cell surface marker of P1CTL from mice that had rejected B7-1$^+$ tumors and found that the P1CTL persisted in mice after rejection of the J558-B7 tumors (Fig. 2*A*, *left column*). As shown in Fig. 1, of the mice that rejected the B7-1$^+$ tumors, some never developed tumors, whereas others developed and rejected the tumors. As expected, P1CTL from those mice that had transient tumor growth displayed a higher level of CD44 and had a higher proportion of CD62L^{low} cells. Moreover, the number of cells with an effector cell marker (26) (1B11, the 130-kDa form of CD43) was also higher. In the group of mice that never developed tumors, however, T cells had a partial activation phenotype. After a short term in vitro restimulation, spleen cells harvested from all recipient mice developed P1A-specific cytotoxicity, although T cells from mice with transient tumor growth were somewhat more cytotoxic.

The failure of P1CTL to reject the J558-Neo tumors can be due to a lack of CTL maturation in the local tumor environment (inductive phase) or to a requirement for B7-1 at the effector phase. We have recently demonstrated that P1CTL can be activated regardless of B7-1 expression on the tumor cells (27). These results suggest that the failure to reject the B7-1$^-$ tumors was not due to a lack of T cell activation in vivo. To bypass the requirement for B7-1 at the inductive phase, we mixed the J558-B7 and J558-Neo cells before injection into RAG-2-deficient mice, which then received purified P1CTL i.v. In RAG-2-deficient mice that received no T cells, tumors grew progressively, and all mice became moribund within 3 wk after tumor inoculation (Fig. 3). However, in mice that received P1CTL, significant tumor retardation was observed. In two separate experiments involving different numbers of P1CTL, we observed significant retardation in both the onset and growth kinetics of tumors. Nevertheless, the majority of the mice

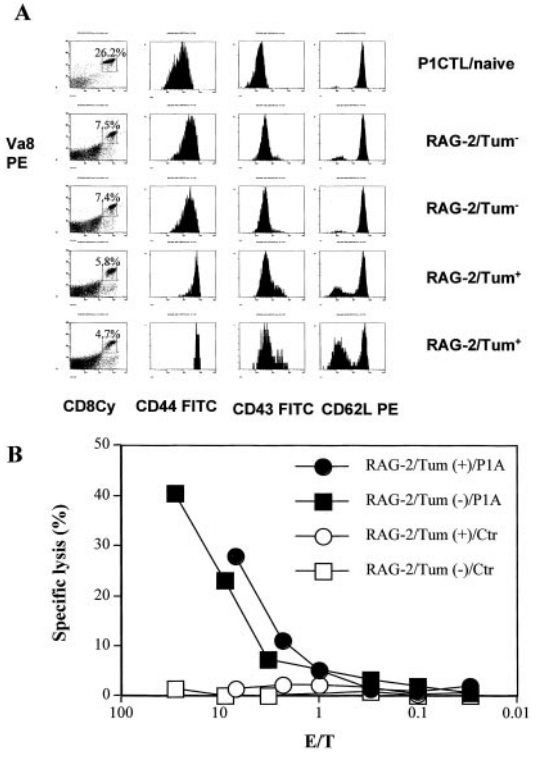

FIGURE 2. Persistence, phenotypes, and recall cytotoxicity of T cells from mice that had rejected the J558-B7 tumors. Purified P1CTL transgenic T cells (5×10^6) were injected into each RAG-$2^{-/-}$ BALB/c mouse i.v. followed by the injection of 5×10^6 J558-B7 tumor cells in the left flank s.c. Tumor growth was monitored daily, and mice were sacrificed at day 30 of injection. As shown in Fig. 1, some mice developed tumors transiently (Tum$^+$), whereas others never developed tumors (Tum$^-$). *A*, Persistence and phenotypes. *Left column*, Frequency of P1CTL (Vα8$^+$CD8$^+$). Two *middle columns*, CD44 and CD43 expression of gated CD8$^+$Vα8$^+$ T cells; *right columns*, expression of CD62L among gated CD8$^+$ T cells. Sources of spleen cells: P1CTL/naive, spleen cells from naive P1CTL transgenic mouse; RAG-2/Tum$^-$, spleen cells from mice that never developed tumors; RAG-2/Tum$^+$, spleen cells from mice that developed and then rejected tumors. *B*, Short term recall cytotoxicity. Spleen cells pooled from mice used in Fig. 2*A* were cultured in vitro with P1A peptide (0.1 μg/ml) for 12 h. Then cells were used as effectors to kill P1A peptide-pulsed P338D1 target cells. Ctr, Control.

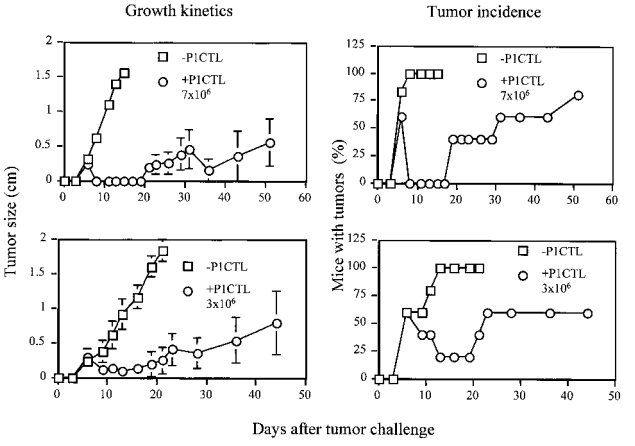

FIGURE 3. Selective rejection of the B7-1$^+$ tumor cells was not due to preferential T cell activation. J558-B7 and J558-neo tumor cells were mixed at a 1:1 ratio, and 5×10^6 cells of each type were injected into the left flank of each mouse. The mice either were left untreated or were treated with 7×10^6 (*top*) or 3×10^6 (*bottom*) of purified CD8$^+$ P1CTL-transgenic T cells i.v. at the same time. Tumor growth kinetics (*left*) and incidence (*right*) were monitored after tumor inoculation.

did eventually develop tumors, and the tumors did progress, albeit at a reduced rate, until euthanasia became necessary.

We isolated tumors from the mice with and without P1CTL treatment and analyzed the expression of B7-1 on the tumor cells. One part of the tumor samples was used for immunohistochemistry, whereas the other part was used to prepare single-cell suspensions for flow cytometry. As shown in Fig. 4, flow cytometry revealed that tumor cells isolated from all J558-Neo tumor cells were B7-1$^-$, and all J558-B7 tumor cells were B7-1$^+$, as expected. In control mice that were not treated by P1CTL, there was a slight dominance of the B7-1$^+$ tumor cells. Thus, expression of B7-1 on the tumor cells did not hinder their growth in the absence of T cells. In mice that received P1CTL, however, essentially all the surviving tumor cells were B7-1$^-$. Immunohistochemistry revealed good microchimerism between the B7-1$^+$ and B7-1$^-$ tumor cells in untreated mice. However, in the P1CTL-treated mice, all but a few surviving tumor cells expressed B7-1 molecules in vivo. These B7-1$^+$ cells were not found in the single-cell suspension prepared from the same sample, perhaps because they were being destroyed in vivo. The fact that many of the B7-1$^-$ tumor cells were not eliminated despite their proximity to the B7-1$^+$ tumor cells indicates that B7-mediated enhancement of tumor destruction in vivo is highly localized.

We have reported that tumor Ag P1A is expressed on multiple lineages of tumors, including plasmacytoma J558, fibrosarcoma Meth A, and mastocytoma P815 (28). In contrast to J558, both Meth A and P815 are metastatic. We tested their susceptibility to P1CTL-mediated tumor rejection. As shown in Table I, in wild-type mice, B7-1$^+$ but not the B7-1$^-$ P815 and Meth A tumors are rejected when they are injected separately in the same mouse. When the two types of tumor cells were injected as a mixture, all recipient mice developed tumors. To determine the cellular origin of the tumor cells, the tumor cells were isolated ex vivo and analyzed for their cell surface expression of B7-1 by flow cytometry. The results indicated that all the tumor cells that had survived in vivo were B7-1$^-$. Thus, introduction of B7-1$^+$ tumor cells in the vicinity of B7-1$^-$ tumor cells did not convey full protection to their B7-1$^-$ tumor cells. Taken together, our data indicate that cognate destruction of multiple lineages of P1A-expressing tumors depends on B7-1 expressed on the tumor cells.

B7-1 on the tumor cells conveys susceptibility of large tumors to CTL therapy

Our data, presented in the above section, indicate that expressing costimulatory molecules on the tumor cells can enhance the effector function of T cells in vivo. The most difficult obstacle for T cell therapy is the resistance of large tumors to T cell treatment. To test whether expression of B7-1 on the large tumors makes them more susceptible to CTL-mediated tumor therapy, we injected either J558-Neo or J558-B7 tumors into the RAG-$2^{-/-}$ mice. When the tumors reached 1.1–1.5 cm, we injected 10×10^6 P1CTL i.v. into the tumor-bearing mice. As shown in Fig. 5*A*, administration of transgenic P1CTL resulted in a significant improvement in the survival of tumor-bearing mice. Thus, untreated mice became moribund within a week after the treatment had started in other groups, while all but one in each group of treated mice survived >4 wk after treatment. However, 60% of mice bearing J558-B7 tumors survived >12 wk; all mice with the J558-Neo tumors succumb within this period (Fig. 5*A*). The treatment did not eliminate the J558-B7 tumors, given that the tumor recurred in all mice (data not

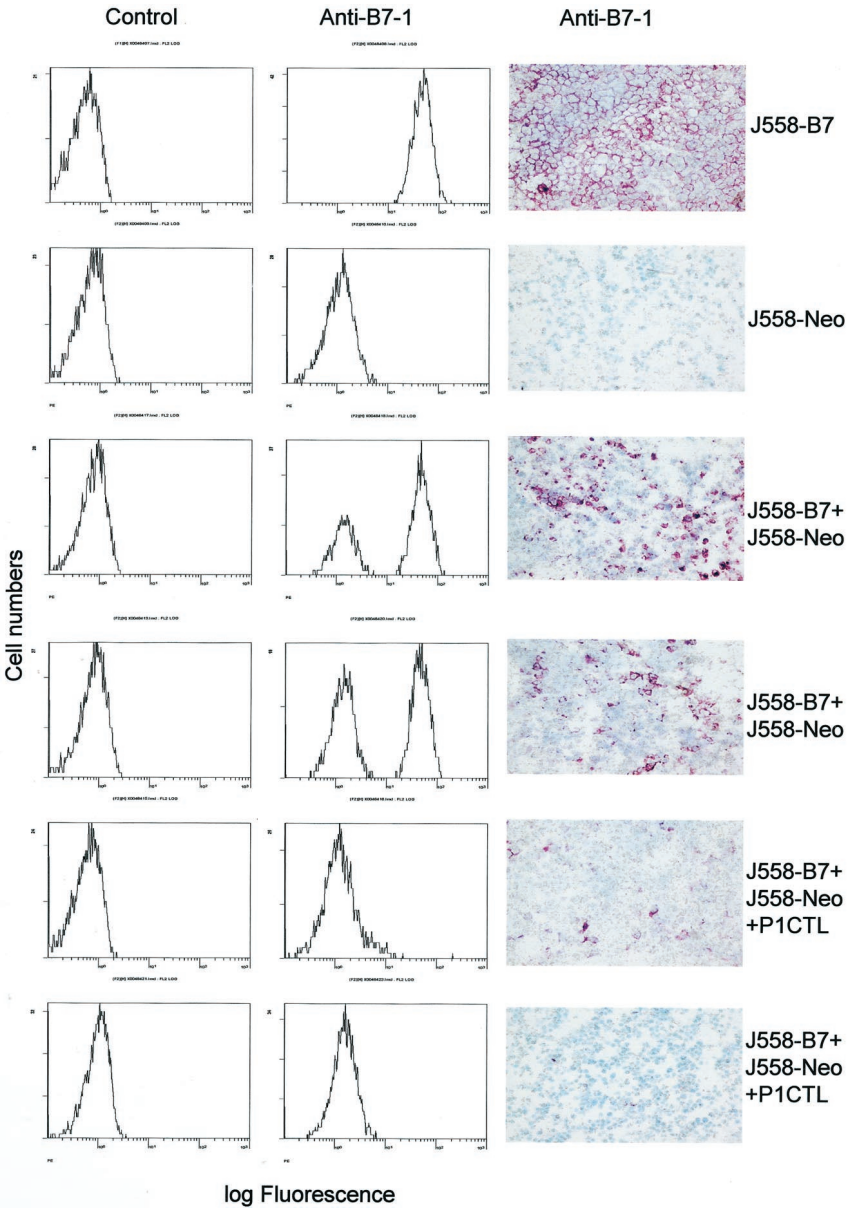

FIGURE 4. Selective elimination of $B7^+$ tumor cells by P1CTL from a mixture of $B7^+$ and $B7^-$ tumor cells. *Left* and *middle columns*, Flow cytometric analysis of the composition of $B7^+$ and $B7^-$ tumors in the presence or absence of P1CTL. Briefly, single-cell suspensions were prepared from freshly isolated tumors and stained with biotinylated anti-B7-1 mAb (*middle column*) or second-step reagent only as control (*left column*). *Right column*, Immunohistochemical analysis of microchimerism of $B7^+$ and $B7^-$ tumors. Tumors derived from J558-B7, J558-Neo or a mixture of the two cell types in RAG-$2^{-/-}$ mice were surgically removed when they reached ~1 cm in diameter, and frozen sections were stained by anti-B7-1 mAb. Data from two representative mice in the groups involving a mixture of J558-B7 and J558-Neo tumors were provided.

shown). Although the mice with recurrent J558-B7 tumors remained healthy at 12 wk after tumor cell injections, the tumor burdens had made euthanasia necessary. These results indicated that whereas the P1CTL are of some therapeutic value for vector-transfected tumors, expression of B7-1 on the tumor cells made the large tumors substantially more susceptible to CTL therapy.

To avoid variation in the inductive phase of CTL response, we injected the J558-Neo and J558-B7 tumors into separate flank of the same RAG-$2^{-/-}$ mice and allowed the tumors grow to ~1.0–1.5 cm in diameter; then 5×10^6 purified P1CTL were injected into each mouse i.v. As shown in Fig. 5*B*, the J558-B7 tumors were rapidly destroyed in all four mice within 10 days after T cell adoptive transfer. Although all of the J558-Neo tumors remained substantially viable, areas of scars and tissue death were found in the J558-Neo tumors (Fig. 5*C*, *top*). This result demonstrated that some immune rejection was also under way in the J558-Neo tumors. We also examined the effect of immunotherapy by H&E staining of tumor tissues. As shown in Fig. 5*C*, in the mice that received P1CTL, no viable cells were observed throughout the sections of all four tumors. Although substantial cell death was also observed in all of the J558-Neo tumor sections, each of the tumors still consisted of a large number of viable tumor cells. These results revealed at the cellular level that large J558-B7 tumors are more susceptible to the P1CTL than were the J558-Neo tumors in the same mice. Preferential rejection of the J558-B7 tumors over the J558-Neo tumors in the same mice clearly demonstrated that local expression of B7-1 increased tumor susceptibility to CTL therapy.

An issue of critical importance for tumor immunotherapy is whether the requirement for local costimulation can be bypassed by in vitro activation of tumor-specific T cells. To address this issue, we adoptively transferred preactivated P1CTL to mice with J588-Neo and J558-B7 tumors ~1.0–1.5 cm in diameter. The activated P1CTL T cells had strong cytotoxicity against P1A-peptide-pulsed targets (Fig. 6*A*). Despite the strong cytotoxicity, they still showed preference in rejecting the $B7\text{-}1^+$ tumors. Thus, the J558-B7 tumors started to shrink at ~3 days after T cell therapy, and the tumors remained small at the time when the mice had to be sacrificed due to the large size of the J558-Neo tumors. The response of J558-Neo tumors was both delayed and transient (Fig. 6*B*). Thus, preactivation of tumor-specific T cells was insufficient to bypass the requirement for local costimulation by B7-1.

Table I. *B7-1-dependent rejection of P1A-expressing P815 and Meth A tumors*[a]

Host	Tumor[b]	Left	Right	P1CTL[c]	Tumor Incidences[d] Left	Right
CBY_{F1}	P815	$B7^-$	$B7^+$	+	12/15	0/15
CBY_{F1}	P815	$B7^-$ + $B7^+$		−	7/8[e]	
$BALB/c^{RAG2^{-/-}}$	P815	$B7^-$	$B7^+$	−	10/10	10/10
$BALB/c^{RAG2^{-/-}}$	P815	$B7^-$	$B7^+$	+	8/8	0/8
$BALB/c^{RAG2^{-/-}}$	P815	$B7^-$ + $B7^+$		−	6/6	
$BALB/c^{RAG2^{-/-}}$	P815	$B7^-$ + $B7^+$		+	6/7[e]	
$BALB/c^{RAG2^{-/-}}$	Meth A	$B7^-$	$B7^+$	−	4/4	4/4
$BALB/c^{RAG2^{-/-}}$	Meth A	$B7^-$	$B7^+$	+	4/4	0/4
$BALB/c^{RAG2^{-/-}}$	Meth A	$B7^-$ + $B7^+$		−	4/4	
$BALB/c^{RAG2^{-/-}}$	Meth A	$B7^-$ + $B7^+$		+	4/4[e]	

[a] Data presented are the summary of two to three experiments.
[b] $B7\text{-}1^+$ or $B7\text{-}1^-$ Meth A (1×10^6) or P815 (2×10^6) tumor cells in 100 μl of serum-free medium were injected s.c. in either the right or left flanks.
[c] CD8 T cells were purified from the pooled spleens and lymph nodes of the P1CTL-transgenic mice and were injected i.p. on the same day of s.c. tumor injection.
[d] Mice were examined physically every other day, and all mice were observed until euthanasia became necessary, usually 4–6 wk after tumor injection. In the $BALB/c^{RAG2^{-/-}}$ mice, all mice that were scored as tumor free never developed tumors, whereas all palpable tumors grew progressively until euthanasia. In the CBY_{F1} mice, however, the $B7^+$ tumors became palpable within 1 wk but were rejected the following week. Tumor incidences are number of mice with tumor per number of mice injected, as determined at the time of sacrifice, usually 4–6 wk after tumor injection.
[e] For the majority of the cases, the tumors derived from a mixture of $B7^+$ and $B7^-$ cells were isolated, and after a short term culture, the B7 phenotypes were determined by flow cytometry. Essentially all tumor cells that grew were $B7^-$.

At 3 days after T cell injection into mice with large tumor burdens, spleen cells and tumor-infiltrating cells from each group were isolated, and total RNA were purified; we analyzed the expression of two groups of genes by RNase protection assay. As shown in Fig. 7*A*, genes involved in regulation of apoptotic cell death were expressed at essentially the same levels among T cells isolated from mice with the J558-B7 and J558-Neo tumors, regardless of whether spleen cells or tumor-infiltrating lymphocytes (TIL)[3] was used. Similarly, expressions of TNF-α, IL-6, IFN-β, and TGF-β1 among the spleen T cells of mice bearing either J558-B7 or J558-Neo tumors were comparable. In contrast, TIL from J558-B7 tumors expressed substantially more IFN-γ mRNA (Fig. 7*B*). The enhanced IFN-γ expression indicated that B7-1 on the tumor cells provided local costimulation for P1CTL.

Effect of anti-CD28 and anti-CTLA4 Abs on the therapy of tumors by P1CTL

The data in *B7-1 on the tumor cells conveys susceptibility of large tumors to CTL therapy* demonstrate that local costimulation promotes CTL-mediated rejection of large tumors. Although it is practically impossible to express B7-1 on all tumor cells, B7-1-mediated signaling can potentially be achieved by using mAbs specific for the B7 receptors, CD28 and CTLA4 (29, 30). We therefore tested the effects of intratumor injection of anti-CD28 Ab 37N and anti-CTLA4 Ab 4F10 on P1CTL-mediated adoptive therapy of tumor. J558-Neo tumor cells were injected into the $RAG\text{-}2^{-/-}$ BALB/c mice that were left untreated until the tumors reached diameters of 1–1.5 cm (Fig. 8). At this point, the mice received 5×10^6 purified $CD8^+$ P1CTL transgenic T cells i.v. On days 0 and 16 of T cell transfer, we injected control hamster Ig, anti-CD28 mAb 37N, or anti-CTLA4 mAb 4F10 into the tumor cells and monitored the growth of the tumors. As shown in Fig. 8, tumor growth was comparable in the groups that received either control IgG or anti-CTLA4 mAb 4F10. However, anti-CD28 mAb 37N substantially prolonged the regression of the J558-Neo tumors. These results suggest that the effector function of B7-1 can be achieved by local administration with anti-CD28 mAb.

[3] Abbreviation used in this paper: TIL, tumor-infiltrating lymphocytes.

Discussion

One of the most puzzling phenomena in tumor immunology is the coexistence of cancers and their specific CTL (7–10). Although it is unclear when antitumor CTL responses are initiated in cancer patients, our studies in animal tumor models indicated that the induction of antitumor CTL response in the host remained suboptimal until the tumors had grown into large sizes (X.-F. Bai, P. Zheng, and Y. Liu, unpublished observations). Thus, failure of CTL to reject large tumors not only explains the poor antitumor CTL immunity but also represents the most difficult challenge in tumor immunotherapy. In the current study, we used transgenic T cells specific for a natural tumor Ag P1A and multiple lineages of P1A-expressing tumors to investigate the effect of local costimulation on the in vivo function of tumor-specific CTL. The results demonstrated that regardless of the tumor sizes, local costimulation substantially increased the efficacy of CTL therapy.

Numerous studies have documented that expression of B7-1 on the tumors enhances tumor rejection (11, 13, 14, 31). The mechanisms for this important function have not been clearly elucidated. It was originally suggested that B7 enhance antitumor CTL response by solely promoted priming of tumor-specific T cells. Although the role for B7-1 in promoting T cell priming has been clearly demonstrated (11, 27, 32, 33), it is unlikely that this is the only mechanism in that T cell priming can take place without direct stimulation by tumor cells. We and others have shown that B7-1 can play an important function for the effector function of antitumor and autoreactive T cells (14, 19, 21). In a model that involved a single clone of T cells, here we show that transgenic CTL specific for tumor Ag P1A preferentially rejected $B7\text{-}1^+$ tumor cells when $B7\text{-}1^+$ and $B7\text{-}1^-$ tumor cells were injected into the same $RAG\text{-}2^{-/-}$ mice. Because this study involved transgenic T cells specific for a single P1A peptide and multiple lineages of P1A-expressing tumors, it offered the strongest in vivo evidence to date for the notion that local costimulation can enhance the effector function of antitumor CTL (9, 14, 19, 21).

Two interesting issues remain to be resolved. First, what is the mechanism for B7-1 to enhance effector function of tumor-specific T cells? We showed that B7-1 enhanced cytolysis of J558-cells.

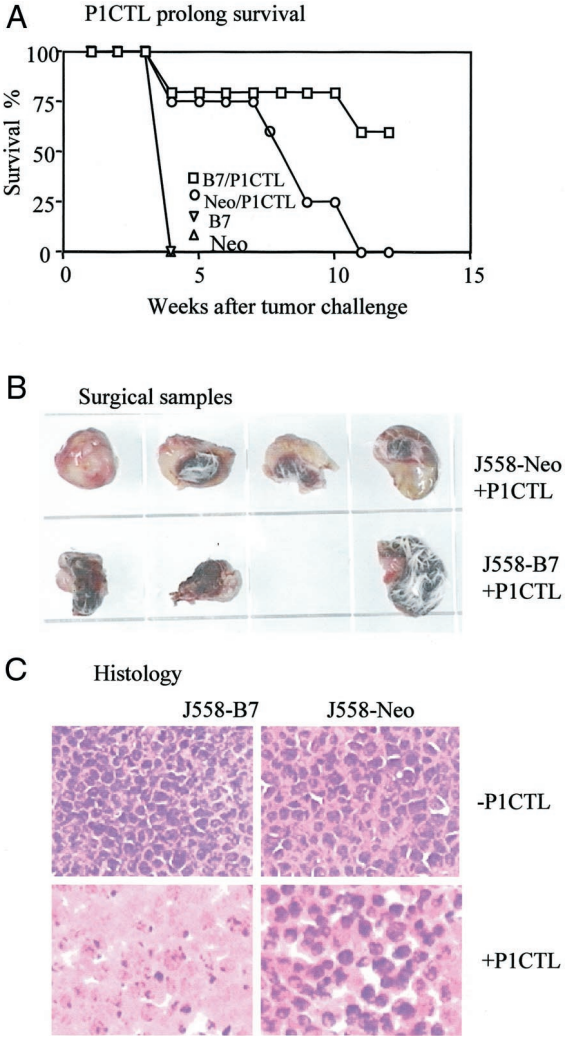

FIGURE 5. Local costimulation promotes tumor rejection. *A,* Survival of mice bearing either J558-B7 or J558-Neo tumors in the presence or absence of P1CTL. RAG-$2^{-/-}$ BALB/c mice were injected with either 5×10^6 J558-B7 or J558-neo tumor cells. At 3 wk after tumor cell injection, when the tumors reached an average of 1.2 cm (J550-Neo tumors) or 1.5 cm (J558-B7 tumors), 10×10^6 purified $CD8^+$ P1CTL-transgenic T cells were injected i.v. Mice were sacrificed either when they were moribund or when the tumor reached 5% of body weight. *B* and *C,* P1CTL preferentially destroyed established B7-1^+ tumors in mice that bore both J558-B7 and J558-Neo tumors. J558-B7 and J558-Neo tumor cells (5×10^6) were injected into separate flanks of the same RAG-$2^{-/-}$ BALB/c mouse; when the tumors grew to ~1.5 cm at 3 wk, 5×10^6 purified $CD8^+$ P1CTL-transgenic T cells were injected. Tumor tissues were harvested at 10 days after T cell therapy. Four mice bearing comparable J558-Neo and J558-B7 tumors were selected for the studies. At the time of harvest, one J558-B7 tumor had been rejected and fallen off the skin. *C,* Histology of a representative J558-B7 tumor and a J558-Neo tumor from an untreated and a P1CTL-treated mice.

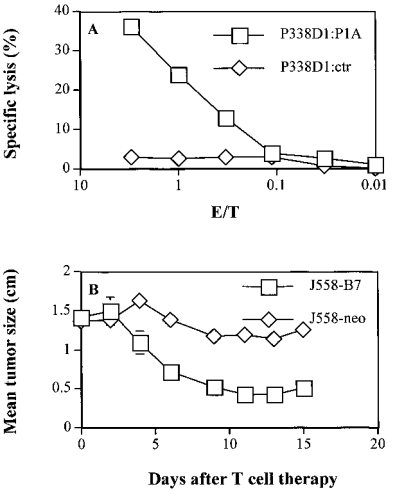

FIGURE 6. Activated T cells preferentially destroy established J558-B7 tumors. RAG-$2^{-/-}$ BALB/c mice were first challenged with J558-B7 (left flank) and J558-neo (right flank) tumors. When the tumors reached 1.5 cm, 5×10^6 activated P1CTL were injected i.v. The tumor size was monitored every other day. *A,* Cytotoxicity of P1CTL before adoptive transfer. *B,* Tumor growth kinetics after adoptive transfer of activated T cells on day 20 after tumor injection. ctr, Control.

Although this can explain the increased rejection of J558-B7 tumors, it cannot explain the preferential rejection of P815-B7 and Meth A-B7, because the two lines are no more sensitive than their parental B7-1^- tumors (data not shown). As an alternative hypothesis, Wu et al. (19) suggested that B7-1 can enhance NK-mediated cytolysis which in turn facilitate priming of tumor-specific CTL. Although the role for B7-1 in NK-mediated cytolysis has been confirmed by several groups (34, 35), it remains to be demonstrated whether NK cells facilitate T cell priming. Moreover, because B7-1^+ and B7-1^- tumors in the same mice have different responses to T cells in the same environment, a systematic mechanism as suggested by Wu et al. (19) would not explain our finding. The fact that the function of B7-1 is almost cognate appears to argue against the importance of IFN-γ up-regulation in TIL of B7-1^+ tumors. However, this possibility cannot be ruled out because cytokine secretion is focused on the area of TCR engagement (36), and as a result, the $B7^+$ tumor cells would be in an environment of higher IFN-γ production.

The second issue is the implication of a role for B7 at the effector phase for tumor immunotherapy. This concept explains the slow progress in T cell based tumor immunotherapy. Alternatively, a critical role of B7 at the effector phase of T cell response provides an opportunity to increase the efficacy of cancer immunotherapy. We show here that expression of B7-1 on the tumors cells made large tumors highly susceptible to CTL therapy. However, the tumors did recur over a 10-week period, and some of the recurrent tumors lost either MHC class I or the transduced B7-1 molecule (data not shown). Thus, even if one assumes 100% efficiency of B7-1 transduction, genetic instability of tumor cells may allow tumor escape of CTL. The genetic heterogeneity can be exacerbated in large tumors, because more variants can accumulate with increasing tumor mass. Indeed, our preliminary analysis favors the notion that alteration of tumors rather than T cells is responsible for tumor recurrence, because a transfer of fresh T cells does not halt the growth of recurrent tumors. Alternatively, one may attempt to bypass such a requirement by activating B7 receptors, CD28 and CTLA4. We showed here that activating CD28 substantially enhanced the antitumor effects of P1CTL adoptive therapy, as indicated by the prolongation of mouse survival and the reduction of tumor size. Anti-CD28 mAb has been used to induce antitumor T cell response in vivo with mixed results (13, 15); our study demonstrates for the first time that the mAb enhanced local costimulation and made large tumors amenable to T cell-based immunotherapy. At face value, the effect of anti-CD28 on tumor rejection of $B7^-$ tumors and the role for B7-1 in cognate destruction of tumor cells in vivo appear at odds with each other. However, anti-CD28 can mimic the function of B7-1 at the

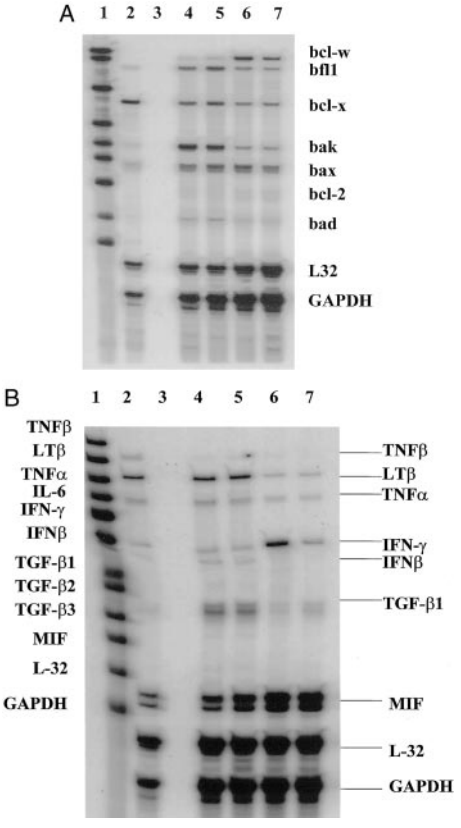

FIGURE 7. RNase protection assays for expression of cytokine genes and those that regulate apoptosis. RAG-$2^{-/-}$ BALB/c mice were challenged with either J558-B7 or J558-Neo tumor cells. When the tumors reached a size of ~1.2 cm, 5×10^6 of purified P1CTL were injected i.v. Three days after injection, spleen cells or tumor-infiltrating cells were isolated, and RNA was purified. *A*, Expression of genes related to apoptotic cell death. *B*, Cytokine gene expression. *Lane 1*, labeled probes; *lane 2*, positive control, *lane 3*, negative control; *lane 4*, spleen cells from J558-B7 tumor-bearing mice; *lane 5*, spleen cells from J558-Neo tumor-bearing mice; *lane 6*, J558-B7 tumor-infiltrating cells; *lane 7*, J558-Neo tumor-infiltrating cells, MIF, Macrophage migration inhibitory factor.

site and time of TCR engagement by B7-1^- tumor cells, whereas a given T cell is unlikely to engage B7-1^+ and B7-1^- tumor cells at the same time.

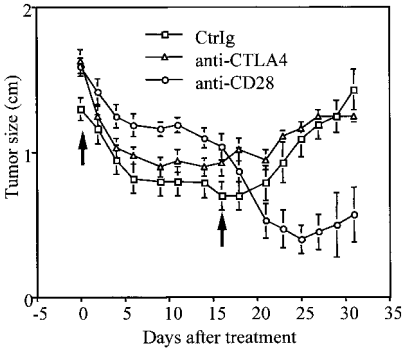

FIGURE 8. Local activation of B7 receptor CD28 promotes tumor rejection by P1CTL. J558-neo tumor cells (5×10^6) were injected into each RAG-$2^{-/-}$ BALB/c mouse; after the tumors reached 1–1.5 cm, 5×10^6 purified $CD8^+$ P1CTL were injected i.v. into each mouse; 4 days later, groups of mice received intratumor injection of either 100 μg hamster IgG ($n = 6$) or 100 μl anti-CTLA4 Ab 4F10 ($n = 5$) or anti-CD28 Ab 37N ($n = 5$), and tumor growth kinetics were monitored. Arrows indicate timing of intratumor injection with Abs. Ctr, Control.

To demonstrate the function of CTL alone, we have chosen a model involving only one clone of T cells without the participation of other subsets of lymphocytes, including the CD4 T cells that can play a major role in maintaining the function of CD8 T cells (37). It would be of interest to further investigate whether local costimulation may offer more beneficial effects in models that involve polyclonal T cells and immunological help from CD4 T cells. Nevertheless, the requirement for B7-1 at the effector phase was initially described (14) and substantiated (19) in models where CD4 T cells are present. It is therefore important to bear in mind that this requirement is not an artifact due to a lack of CD4 T cell help.

Surprisingly, anti-CTLA4 mAb 4F10 did not increase the therapeutic efficacy of P1CTL if the mice bore only J558-Neo tumors, in sharp contrast to our recent observation that the same mAb caused rejection of the J558-Neo tumor if the mice also bore a J558-B7 tumor (data not shown). These two observations can be reconciled if B7-1 on the tumor cells and anti-CTLA4 mAbs act synergistically in promoting the effector function of antitumor immunity.

One of the most perplexing observations in cancer immunology, and certainly one of the most difficult obstacles to cancer immunotherapy, is the poor effector function of cancer-specific T cells, especially when the tumor burden is large. Our current studies indicate that effector function is reinvigorated by local costimulation, which leads to a substantial increase of the efficacy of CTL therapy for large tumors in vivo. Because cancer-specific T cells are present in cancer patients at high numbers (7, 8), increasing their effector function by local costimulation as demonstrated here, may broaden the horizon of T cell-based cancer immunotherapy.

Acknowledgments

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