Maturation of Cytotoxic T Lymphocytes against a B7-transfected Nonmetastatic Tumor: A Critical Role for Costimulation by B7 on Both Tumor and Host Antigen-presenting Cells

Maja Maric, Pan Zheng, Supria Sarma, Yong Guo, and Yang Liu

Michael Heidelberger Division of Immunology, Department of Pathology and Kaplan Comprehensive Cancer Center, New York University Medical Center, New York, NY 10016

ABSTRACT

It is generally believed that CTLs mature in lymphoid organs and then migrate into target tissues to execute their effector functions. This notion, however, is based on studies using antigens that are readily localized in the lymphoid tissue, such as viruses and allogeneic transplants. The site for maturation of CTLs for nonmetastatic tumors has not been determined. Because nonmetastatic tumor cells are not localized in lymphoid tissues, it is questionable whether such tumors are efficient inducers of antitumor CTLs. Here, we report that a nonmetastatic B7+ plasmacytoma induces strong effector CTL response. Thus, it is possible to induce CTLs with strong ex vivo CTL activity in the absence of tumor metastasis. In addition, a detailed kinetic analysis of CD8 T cell recruitment and maturation of CTL activity suggests that antitumor CTLs mature within the tumor rather than in the lymphoid tissues. Interestingly, despite B7−1 expression on tumor cells, induction of effector CTLs also requires costimulation by B7 on host antigen-presenting cells. These findings have important implications for tumor gene therapy and for understanding the mechanism of CTL induction in vivo.

INTRODUCTION

Classical experiments by Barker and Billingham (1) established that intact draining lymphatics are essential for sensitization of T lymphocytes for rejection of allogeneic grafts. Since then, several other lines of evidence have corroborated the notion that the secondary lymphoid organs, the lymph nodes and spleen, are the sites for maturation of CTLs. For instance, direct injection of antigen-bearing cells into the spleen significantly reduced the antigen doses required for CTL priming (2). In addition, in vivo administration of anti-L-selectin mAb, which blocks homing of naive T cells to lymph node, substantially reduced the production of T helper cells and precursors for CTLs (3, 4). It is generally perceived that to induce CTLs (3, 4) the lymphoid organs (5). Although tumor antigens can be localized in the lymphoid tissues by a process analogous to cross-priming (6), a recent study revealed that direct localization of tumor cells into lymphoid organ, a process that mimics tumor metastasis, is the most efficient way to prime T cells (2). Because metastasis occurs at late stage of tumor development, immunity induced by metastatic tumor cells in the lymphoid organ may not be effective enough to eradicate disseminated tumors, either because of tumor burden, or because of increased malignancy of the metastatic tumors. Exploring conditions under which antitumor CTLs can be induced in the absence of tumor metastasis may provide new insight in tumor immuno-therapy.

It is well established that activation of T cells requires two types of biological signals (7–12). Signal 1 is delivered by antigen, the complex formed between the MHC molecules and antigenic peptides. Signal 2 is delivered by costimulatory molecules, of which B7 family members B7−1 and B7−2 are known as prototypes (13–18). Although most tissues express MHC class I and have the necessary machinery to present antigen to T cells, constitutive or inducible expression of costimulatory molecules seems to be limited to APCs that reside primarily in lymphoid tissues (19–21). It is, therefore, possible to explain the lymphoid localization of CTL response based on the requirement for costimulatory molecules on APC.

Expression of costimulatory molecules such as B7−1, B7−2, and the heat-stable antigen on a variety of tumor models, enhances T cell immunity (22–26). A critical issue that has not been directly addressed is whether expression of costimulatory molecules allows the maturation of antitumor CTLs within the tumor. Two factors complicate studying where maturation of antitumor CTLs takes place. First, tumor cells may metastasize into lymphoid tissue, thus making it difficult to assess the site at which tumor cells encounter T lymphocytes. Second, with a few notable exceptions (25, 27, 28), antitumor CTLs are detectable only after one or multiple rounds of in vitro restimulation. It is, therefore, difficult to determine the site where CTLs with full effector function are produced. To avoid these complications, we have used a plasmacytoma model, J558, which we show here does not metastasize into local lymph node. More importantly, ex vivo TILs from B7−expressing tumors have potent cytotoxicity (25), and we have recently defined the major epitope for TILs as the P1A peptide 35–43 presented by Ld (28). We, therefore, decided to use this model to address the following questions: (a) is it possible to induce CTLs for nonmetastatic B7−1-expressing tumors?; (b) where is the site for maturation of antitumor CTLs?; and (c) what is the contribution of costimulatory molecules B7 on the tumor versus those on host APC?

MATERIALS AND METHODS

Cell Lines, Antibodies, and Experimental Animals. Plasmacytoma J558 transfected with either murine B7−1 (J558-B7) or vector alone (J558-Neo) has been described previously (25). Macrophage cell line P388D1 (H-2b) was obtained from American Type Culture Collection (Manassas, VA). They were cultured in RPMI medium containing 5% FCS and 100 μg/ml penicillin and streptomycin.

Antibodies used in this study were: anti-PC.1 mAb 4G6 (29), anti-B7−1 mAb 3A12 (18), 10.16A.1 (30), anti-B7−2 mAb GL-1 (17), and phycoerythrin-labeled-anti-CD8 mAb (PharMingen, San Diego, CA). BALB/c mice were purchased from the animal facility of the National Cancer Institute (Bethesda, MD). Male mice between 6 and 12 weeks of age were used in all experiments.

The abbreviations used are: APC, antigen-presenting cells; TIL, tumor-infiltrating lymphocyte.
Transfection of Human B7–1 into J558 Cells. Transfection of J558 cells follows the same protocol as described (25), except that human B7–1 cDNA (Ref. 31; kindly provided by Dr. Lee Nadler, Harvard Medical School) was used instead of murine B7–1. After selection by G418, human B7–1 clones were screened by flow cytometry using CTLA4 immunoglobulin (32). Clones 2 and 5 expressing different levels of B7–1 were selected for the current study.

Preparation of TILs. J558-B7 and J558-Neo tumors were obtained by surgery from tumor-bearing mice. Single-cell suspensions were prepared by grinding tumors with frosted end glass slides. Viable cells were isolated by Ficoll-Hypaque solution. To prepare TILs, single cell suspensions from two tumors were pooled and incubated with 1:800 anti-PC.1 mAb ascites for 45 min at 4°C. This was followed by incubation with Goat-anti-Rat IgG-coated iron beads (Biosource International, Weltlake Village, CA). The tumor cells that were coated with the beads were removed by a magnet. The remaining cells were used as TILs for both flow cytometry and cytotoxicity.

CTL Assay. The cytotoxicity of TILs, lymph node cells, and splenocytes was measured in a 6-h 51Cr-release assay, using tumor cell lines J558-B7 and peptide-coated P388D1 as targets. Briefly, unpulsed J558-B7 or P388D1 cells pulsed with either 10 μg/ml P1A antigenic peptide (LYPGLWLVF; Ref. 33) produced by Research Genetics (Huntsville, AL), or a control Kb binding peptide (KYGVSQAQDI) were labeled with 51Cr and incubated with effecter cells. The amount of released 51Cr was determined using a gamma counter, and the percentage of specific lysis was calculated as has been described (25).

In Fig. 2, the E:T ratio is calculated using the number of CD8 T cells in the TILs as that of effectors to directly compare the cytotoxicity of CD8 T cells recovered at different time points. In all other experiments, the number of leukocytes added was used as that of effectors.

Quantitation of CD8 T Cell Infiltration into Tumors. CD8 T cells infiltrated in the tumors were measured by flow cytometry. The number of CD8 T cells were calculated according to the following formula:

Number of CD8 T cells/10^6 tumor cells = Number of TILs from 10 X 10^6 tumor cells X the percentage of CD8 T cells in the TILs. Pools of two to three tumors were harvested at each time point.

Detection of Endogenous B7 Molecules in hB7-1-transfected Tumor Cells in Vivo. To determine whether endogenous B7–1 or B7–2 is induced in vivo, single cell suspension was prepared from freshly isolated tumors, and stained with anti-CD45-PE and mAbs specific for either murine B7–1 or B7–2. Briefly, single-cell suspension was first incubated with anti-B7–1 (10.16A) or anti-B7–2 (GL-1) mAbs. After removing unbound mAbs, appropriate second-step reagents, either FITC-labeled goat anti-hamster IgG (Rat/mouse adsorbed; Caltag, Mountain View, CA), or FITC-labeled mouse anti-rat IgG (Accurate Chemical and Scientific Corp., Westbury, NY), were added and incubated for 45 min. The unbound second-step reagents were removed, and normal rat IgG (500 μg/ml) was added to block unoccupied sites of the second-step reagents on the cells. The PE-conjugated anti-CD45 mAb (PharMingen) was then added to mark the infiltrated leukocytes.

Detection of Tumor Metastasis to Local Lymph Nodes. Either J558-B7 or J558-Neo tumor cells (5 X 10⁶) were injected in the left inguinal of BALB/c mice. On days 3, 7, 10, and 17 after tumor injection, both the tumors and the inguinal lymph nodes were harvested. The presence of tumor cells in the inguinal lymph nodes was determined by histology. Briefly, the inguinal lymph nodes were fixed in 10% formaldehyde overnight. The fixed tissues were embedded in paraffin and the 5-μm tissue sections were stained with H&E. Nine to 18 cross-sections were prepared from each lymph node to search for possible metastasis of tumor cells.

As an alternative approach, we have used PCR to determine whether a small number of tumor cells have infiltrated the draining lymph nodes. We have used neomycin-resistance gene as the tumor marker. Briefly, syngeneic BALB/c mice received injections of 5 X 10⁶ J558-B7 cells in the footpads. The draining popliteal lymph nodes were harvested at 1 week after tumor challenge, when the tumor is palpable at the site of injection. Genomic DNA isolated from draining lymph nodes were amplified using 5'CAAGCTGGCGGTTAGGAC as forward primer and 5'CAGAGGCCTTCAACCCCTG as reverse primer. Each cycle of the PCR reaction consists of 30 s at 94°C, 1' at 50°C and 1' at 68°C. After 40 cycles, the PCR products were separated in 1% agarose gel, transferred into nylon membrane and probed with 32P-labeled neomycin cDNA. The quality of genomic DNA is controlled by amplification of exon 3 of murine proto-oncogene pml (34) using 5'GAAACAGTCTATGGCCTTGAACC as forward primer and CTATGAGCATGGCCTCAACCCTTAC as reverse primers. The PCR products are also quantified by hybridization with 32P-labeled pml cDNA.

In Vivo Antibody Treatment. BALB/c mice were given s.c. injections of either 5 X 10⁶ J558 cells expressing murine B7–1, or 10 X 10⁶ J558 cells expressing human B7–1 (either clone 2 or clone 5). These mice also received i.p. injections of PBS, control immunoglobulin, anti-B7–1 mAb 3A12, and 3A12 + anti-B7–2 mAb (GL-1) on days 3, 6, 9, and 12 after tumor injection, at a dose of 200 μg/mouse/injection. On days 14 and 17, the tumors were obtained by surgery and the CTL activity in the tumors was determined in vitro.

RESULTS

J558-B7 and J558-Neo Tumors Are Not Metastatic: Localization of Tumor Cells in Lymphoid Organs Is Not Essential for Induction of Strong Antitumor CTLs in Vivo. s.c. injection of J558-B7 and J558-Neo tumor cells in the left inguinal of BALB/c mice leads to development of solid tumors, which are palpable in most mice within 7–10 days after tumor injection. Approximately 80% of the J558-B7 tumors regress within 4 weeks, whereas all J558-Neo tumors grow progressively (25). The close proximity of tumors and inguinal lymph nodes has made it easy for us to determine whether the tumors are metastatic.

Although the tumors and the lymph nodes are less than 5 mm apart (Fig. 1), the lymph node is not invaded by the tumors. Nine to 18 consecutive 5-μm sections of each lymph node were examined after H&E staining. Such extensive histological analysis of inguinal lymph nodes obtained on days 3, 7, 10, and 17 after injections of tumor cells have failed to reveal any tumor cells in the lymph nodes. Moreover, we have failed to find tumors, at sites other than that of initial inoculation, in mice that bear massive J558 tumors. These results demonstrate that the J558-tumors used in this study are not metastatic.

Interestingly, local tumor growth leads to activation of both B and T cells in the lymph node, as evidenced by the formation of germinal centers and the expansion of the paracortex (Fig. 1). However, it should be noted that such lymphocyte activation does not depend on B7–1 expression on the tumor cells, as similar degree of lymph node activation is observed in the inguinal lymph node in mice bearing either J558-B7 or J558-Neo tumors (Fig. 1).

We have also used PCR to determine whether tumor cells infiltrate the draining lymph nodes. The transfected neomycin-resistance gene was used as the tumor marker. We expect to achieve high sensitivity because one tumor cell usually contains multiple copies of the drug-resistance gene. To determine the sensitivity of the PCR-reaction, genomic DNA from 1 or 10 tumor cells was added to 10% of that from one draining popliteal lymph node. As shown in Fig. 2 left panels, the PCR is sensitive enough to detect one tumor cell in each reaction. Despite this, it did not detect any tumor cells in five draining lymph nodes from tumor-bearing mice (Fig. 2, right panels). Two additional experiments also fail to detect neomycin-resistance gene in any of these nodes (data not shown). Each PCR reaction sampled 10% of genomic DNA from one lymph node, one negative result, therefore, indicates that the number of tumor cells in one draining lymph node is less than 10. Because all 15 PCR reactions failed to detect any tumor cells, it is unlikely to have tumor cells present in the draining lymph nodes. Thus, the sensitive PCR confirms that J558 tumor is not metastatic.

Because the J558-B7 tumors induce CTLs with a strong ex vivo cytotoxicity (Refs. 25 and 28, and see below), the results presented in this section indicated that lymphoid localization of tumor cells is not essential for induction of antitumor CTL when B7–1 is expressed on tumor cells.
Kinetics and Localization of Antitumor CTL Responses in Vivo.

We have previously demonstrated that B7-1-transfected tumors are infiltrated by a large number of T cells, most of which are CD8+ (25, 35). A significant number of B cells, macrophages, and dendritic cells are also found in the tumor (Ref. 35, and data not shown). In addition, the TILs are cytotoxic to J558-B7, and the effectors are CD8 T cells (25, 28). If all of the infiltrating CD8 T cells have become CTLs before their migration into the tumor, one may expect the cytotoxicity of TILs to correlate with the number of CD8 T cells recovered from tumors, and, on a cell-to-cell basis, CD8 T cells recovered at different time points should have a comparable cytotoxicity. As shown in Fig. 3a, in J558-B7 tumors, infiltration of CD8 T cells is already significant on day 9. The number of CD8 T cells recovered from 10 × 10⁶ tumor cells increases by 10-fold between day 9 and day 12, and remains largely unchanged thereafter. Interestingly, CD8 T cells recovered at early time points are not cytotoxic. The cytotoxicity of the CD8 T cells becomes detectable on day 15, and has increased by 30- to 100-fold between day 15 and day 22 (Fig. 3b). In multiple experiments, although the onsets of CTL response differ somewhat (see Fig. 4 for an example), infiltration of CD8 T cells invariably precedes the accumulation of effector CTLs by 5-7 days. These results do not support the notion that all CD8 T cells have differentiated into effector CTLs before their migration into tumors. The number of CD8 T cells is lower in J558-Neo tumor, and the CD8 T cells are generally not cytotoxic to tumors. This is consistent with our previous observation that expression of costimulatory molecule B7-1 is required for the sustained predominance of CD8 T cells within a tumor (25, 35). In addition, a comparison of the CTL response in J558-B7 and J558-Neo tumors reveals a critical role of B7 in antitumor CTL responses.

One interpretation of these results is that the CD8 T cells do not mature into CTLs until they have reached B7-expressing tumors. However, it is also possible that antitumor CTLs are produced in secondary lymphoid organ, and then migrate into tumors. To differentiate between these possibilities, we searched for antitumor CTLs in local lymph nodes or spleens. As shown in Fig. 4, although antitumor CTLs are detectable within a tumor approximately 2 weeks after tumor inoculation, they are not detectable in spleen and lymph nodes, on, or before that date, even if 100-fold more spleen or lymph node cells are used for the CTL assay. Most notably, although a rapid increase in effector activity is detected within TILs between day 13 and day 14, no concurrent increase of CTLs is detected in lymphoid organs. The abrupt increase of cytotoxic T cells within the J558-B7 tumors favors a local maturation event rather than a clonal expansion.
Fig. 2. Detection of tumor cells within draining lymph nodes by PCR. Left panels, sensitivity of the PCR. The templates consist of DNA from 10, 1, or 0 tumor cells and 10% of genomic DNA isolated from a popliteal lymph node. After amplifications with primers for either neomycin-resistance gene (Neo, top) or pml (bottom) genes, the PCR products were quantitated by Southern blot using 32P-labeled DNA probes. Right panels, J558-B7 tumor cells do not infiltrate draining lymph nodes. Draining popliteal lymph nodes were isolated from mice that have received $5 \times 10^6$ J558-B7 tumor cells in the footpads 7 days previously. Each reaction consists of 10% of genomic DNA from a different draining lymph node. The data are representative of three independent amplification of the five lymph nodes.

Fig. 3. Kinetic analysis of CD8 T cells and antitumor CTLs accumulated within the tumors. Number (a, c) and cytotoxicity (b, d) of CD8 T cells isolated from J558-B7 tumors (a, b) and J558-Neo tumors (c, d) at different time points after tumor challenge. The numbers shown are those isolated per $10^6$ viable tumor cells. Single cell suspensions were prepared from surgically removed J558-B7 and J558-Neo tumors. Two to three tumors were pooled before preparation of single cell suspensions. The PC1 T tumor cells were removed using specific mAb. The remaining TILs were used for flow cytometry and cytotoxicity assay using J558-B7 as target cells. The E:T ratio is calculated based on the number of CD8 T cells in the TILs. The same symbols were used for panels b and d.

of effector cells slowly accumulated from the spleen or lymph nodes, although the latter cannot be formally ruled out.

We have previously demonstrated that a major tumor antigen recognized by the effector CTLs within a J558-B7 tumor is an unmutated tumor antigen P1A. To test whether anti-P1A CTLs are produced within tumors, we analyzed the anti-P1A CTL activity of TILs and the secondary lymphoid organs. Much like the antitumor CTL, P1A-specific CTL response is also seen in tumors on days 14, 17, and 19, but not in the spleen and lymph nodes at any of these time points (Fig. 5). On day 19, the TILs are at least 100-fold more efficient than spleen or lymph node cells (Fig. 5e). The complete absence of P1A-specific CTLs in the spleen and lymph nodes suggests that the low level of cytotoxicity toward J558-B7 detected in spleen cells (Fig. 4) is not antigen-specific. Taken together, the results presented in this section support the notion that maturation of the bulk of the antitumor CTLs takes place within nonmetastatic J558-B7 tumors, although it is formally possible that some CTLs may mature in local lymphoid tissues.
Essential Contribution of B7 on Both Tumor and Host APC to Intratumor Maturation of CTLs. Our detailed comparison of antitumor CTL response in mice bearing J558 tumors transfected with either control vector or B7-1 clearly demonstrated an essential role of B7 on tumor for CTL maturation. However, it remains to be determined whether costimulation by host APC is required for antitumor immunity. To achieve selective blockade of host costimulatory molecules, we transfected human B7-1 molecule into the J558 cells. Two transfectants, J558-hB7#2 and #5, expressing different levels of human B7-1 as judged by their binding to CTLA4 immunoglobulin (Fig. 6), were produced for the study. Among them, clone 5 was rejected within 5–7 days, and the antitumor CTL was not detectable in the tumor-bearing mice (data not shown). This is, perhaps, due to the fact that the high level of human B7-1 led to enhanced NK lysis (36, 37). In contrast, clone 2 was not rejected in the first 3 weeks, and significant anti-Pi A CTLs can be detected after 2 weeks. This clone was chosen to address the contribution of host B7 in the induction of antitumor CTLs.

To ensure selective blockade of host APC, we need to ascertain that murine B7-1 and B7-2 were not induced on the host APC. We, therefore, tested if murine B7-1 and/or B7-2 can be induced on the human B7-1-transfected tumors. Because the hB7-1-transfected plasmacytoma does not express CD45 (Fig. 7a), we can use this marker to distinguish tumor and infiltrating host cells. Moreover, murine B7-1 (Fig. 7b), and B7-2 (Fig. 7c) are also absent. Single-cell suspension of the tumor contained substantial amounts of CD45+ leukocytes, some of which expressed murine B7-1 (Fig. 7d) and B7-2. In contrast, CD45+ tumor cells were devoid of murine B7-1 and B7-2. The apparent binding of a small number (2.2%) CD45+ cells by anti-B7-1 mAb is nonspecific, as the tumor cell line cultured in vitro gives similar binding (Fig. 7b). Thus CD45+ tumor cells remain negative for murine B7-1 and B7-2 in vivo, whereas a substantial amount B7+ infiltrating cells are present in the tumor milieu (Fig. 7d and e).

As shown in Fig. 6, human B7-1 does not cross-react with anti-mouse B7-1 mAb 3A12. We, therefore, used this mAb to evaluate contribution of the B7 expressed on host APC. As shown in Fig. 8, in control immunoglobulin-treated mice, significant anti-Pi A CTLs developed within human B7-1-transfected tumor cells. Interestingly, blockade of mouse B7-1 and B7-2 by mAbs completely abrogated the development of antitumor CTLs. These results reveal an essential role of B7-1 and/or B7-2 expressed on host APC, even if B7-1 is present on the tumor cells. Although the lack of antihuman B7-1 mAb, which blocks the human B7-1 in an in vivo setting, has prevented us from confirming the role of human B7-1 by blocking study, the essential requirement for either human or mouse B7 on the tumor cells for CTL response is confirmed by the fact that B7-1 clones cannot induce CTL response (Fig. 3–5 and data not shown).

To rule out the possibility that the low level of human B7-1 expressed on the tumor cells may be responsible for the requirement for B7 on the host APC, we investigated whether host B7-2 is involved in the induction of CTLs when the transfectant expressing a high level of murine B7-1 is used. We injected mAbs to B7-1 and/or B7-2 into mice that received murine B7-1-transfected tumor cells, and measured the CTLs accumulated within the tumors. As shown in Fig. 9a, when a single mAb is used, anti-B7-1 reduced CTL response by 90%. This reduction is manifested by a 2-fold decrease in the number of TILs accumulated within tumors, and a 6-fold decrease in cytotoxicity on cell-to-cell basis. This strong effect can be attributed to the function of B7-1 on tumor and/or host APC. In contrast, anti-B7-2 mAb alone has no effect on the CTL response. Interestingly, in the presence of anti-B7-1 mAb, anti-B7-2 mAb drastically reduced the remaining anti-Pi A CTL response (Fig 9b). Because the tumor cells do not express B7-2 (Fig. 7c), the effect of anti-B7-2 in the presence of anti-B7-1 can be attributed only to the function of B7-2 on the host APC. Thus, costimulation by B7 on host APC is involved in the induction of anti-Pi A CTL despite the high level of B7-1 expressed on the tumor cells.

**DISCUSSION**

Accumulating data have revealed two basic requirements for activation of T cells in vivo, namely lymphoid location of antigen (1–5)
RECRUITMENT AND FUNCTIONAL MATURATION OF CD8 T CELLS

Fig. 5. Kinetic analysis of anti-P1A effector in tumors and in secondary lymphoid organs. BALB/c mice received s.c. injections in the left inguinal with $5 \times 10^6$ J558-B7 or J558 Neo cells. On days 7 (a), 10 (b), 14 (c), 17 (d), and 19 (e) after the injection, left inguinal lymph node and spleen cells, as well as TILs, were isolated and compared for P1A-specific CTLs in a 6-h $^{51}$Cr-release assay. The target used was the P388D1 cell line pulsed with a P1A peptide (AA35-43) or a Kd-binding peptide as negative control. The cells used were pooled from two to three tumor-bearing mice. The number of leukocytes added into the CTL assay was used as that of effectors to calculate the E:T ratio.

and delivery of two biological (antigenic and costimulatory) signals to T cells by APC (7-12). However, it is possible that these two requirements are fundamentally the same. Although cells in most tissues can present antigens in the MHC class I pathway, APC that are capable of either constitutive or inductive expression of costimulatory molecules (19-21) reside primarily in lymphoid organs. Thus, lymphoid localization of antigen allows professional APC to present both antigenic and costimulatory signals to T cells. This hypothesis predicts that expression of costimulatory molecules on tumors, which we showed to allow accumulation of T cells and APC within the tumor (35), should allow activation of antitumor CTLs within the tumor. Alternatively, it is possible that, in addition to antigenic and costimulatory signals, the highly sophisticated architecture of secondary lymphoid organs is necessary for T cell activation (38).

We have used a B7-1-transfected plasmacytoma to study the site for CTL maturation. To our knowledge, this remains the only tumor model in which mature CTLs recognizing a defined tumor antigen can be detected without in vitro restimulation. This unique property allows us to investigate the site in which the mature CTLs are first produced. Surprisingly, our extensive search has failed to reveal mature antitumor CTLs in secondary lymphoid organs, such as local lymph nodes and spleen, even in the same mice in which potent antitumor CTLs are abundantly detected in the tumor infiltrates. In addition, detailed kinetic studies revealed that infiltration of CD8 T cells into tumors usually precedes the maturation of CTLs by 5-7 days. This time span is consistent with what is required for maturation of antiviral CTLs in the spleen after viral infection (39). Furthermore, mature CTLs are not found in the lymphoid organs at, or before, or after the point when CTLs become detectable within tumors. This is in sharp contrast with the findings from experimental models where CTLs were believed to have been produced in lymphoid organs (reviewed in Ref. 5), and thus makes it very unlikely that mature CTLs for nonmetastatic tumor are being generated within the lymphoid organs before accumulation within the tumors. Most importantly, the abrupt increase of CTL activity within the tumor (a more than 30-fold increase between day 13 and day 14 when a very small increase in the number of CD8 T cells is noted) can be explained by maturation of T cells, but not solely by either local clonal expansion or gradual migration of CTLs from the spleen or lymph node, although the latter mechanism may contribute to the CTL activity detected in the TILs. Taken together, although our results do not rule out maturation of some CTLs within lymphoid tissues, the data strongly suggest that the bulk of the antitumor CTLs mature within a B7-1-expressing tumor.

It should be pointed out that the nature of the assays used for the study allowed us to address the site for maturation of antitumor CTLs, but not necessarily the site for initial priming of T cells. It is,
Fig. 6. Antimurine B7-1 mAb 3A12 does not interact with human B7-1. J558 cells transfected with either vector alone (J558-Neo), murine B7-1 (J558-B7), or human B7-1 (J558-hB7-1) were incubated with either medium alone (dotted lines), CTLA4 immunoglobulin (top panels, solid lines), or antimurine B7-1 mAb 3A12 (solid lines, bottom panels). The binding of the anti-B7-1 mAb is detected with FITC-labeled goat-anti-hamster immunoglobulin, and that of CTLA4 immunoglobulin is detected by goat anti-mouse immunoglobulin.

Fig. 7. Human B7-1-transfected tumor cells (J558-hB7) do not express murine B7-1 and B7-2 in vivo. J558-hB7 cells (5 × 10⁶) were injected s.c. in the left inguina, and the tumors were removed 2 weeks later by surgery. Viable single cell suspension from either J558-hB7 cell line (a, b, and c) or ex vivo tumors (d, e) were analyzed for CD45 (a, d, e) and murine B7-1 (b, d) and B7-2 (c, e) expression. Note all B7-1⁺ or B7-2⁺ cells are host infiltrating cells as they express CD45.
Fig. 8. Costimulatory molecules expressed on host APC are essential for maturation of anti-P1A CTLs within a B7-1-expressing tumor. BALB/c mice received s.c. injections of $10 \times 10^9$ of human B7-1-transfected J558 cells (clone 2). On days 0, 3, 6, 9, 12, and 15, the tumor-bearing mice received i.p. injections of control immunoglobulin, or anti-B7-1 mAb 3A12 + anti-B7-2 mAb GL-1. The TILs were isolated on day 14 (a), or day 17 (b), and the CTL activity against P388D1 cells pulsed with either P1A or a control K^b-binding peptide (K^P) determined in a 6-h 5^Cr release assay. The number of leukocytes added was used as that of effectors to calculate the E:T ratio.

Fig. 9. A contribution of B7-2 on host APC to the induction of anti-P1A CTL response within tumors. a, anti-B7-1 alone, but not anti-B7-2 alone, blocks anti-P1A CTL response. BALB/c mice received s.c. injections in the left inguinal with $5 \times 10^9$ J558-B7 cells, on days 0, 3, 6, and 9; the tumor-bearing mice received i.p. injections with PBS, or anti-B7-1 mAb 3A12, anti-B7-2 mAb GL-1. Anti-P1A CTL activity of the TILs was measured on day 14, as described in Fig. 7 legend. b, in the presence of anti-B7-1, anti-B7-2 blocks remaining anti-P1A CTL responses. Detail as a, except that either anti-B7-1, or anti-B7-1 + anti-B7-2 are used. Data presented in a and b are from the same experiment. The number of leukocytes added was used as that of effectors to calculate the E:T ratio.

Therefore, possible that initiation of antitumor immune response has taken place in the lymphoid tissues. However, a recent study (4) showed that homing of naive T cells to peripheral lymph nodes is not required for priming antitumor CTL responses when B7-1-expressing tumors are used, although the same study documented a strict requirement for naive T cell homing to lymph node for priming antitumor CTLs when B7-2 tumor cells were used. Thus, expression of B7-1 can alter the site of T cell priming.

Although tumor antigens can be transported into lymphoid tissue by APC via cross-priming (40), a recent study suggests that localization of tumor cells directly into lymphoid organ is the most efficient way for tumor antigens to localize into local lymph node (2). Because this is achieved normally by tumor metastasis, the general requirement for lymphoid localization for CTL maturation in vivo suggests that optimal activation of antitumor CTL responses can be induced only after tumor metastasis, when the tumors can become less susceptible to immunotherapy. Here we showed that a B7-1-expressing plasmacytoma J558, which induces CTLs with high ex vivo cytotoxicity, is not metastatic. Our results indicate that local expression of costimulatory molecules on the tumor cells bypasses this strict requirement. In situ delivery of costimulatory molecules into tumors may have the potential to induce antitumor immunity before tumor metastasis.

Because the same B7-1 is expressed both on the tumor cells and on the host APC, it is difficult to selectively block B7 expressed on the host APC. To circumvent this problem, we expressed human B7-1 on the J558 tumor cells. In contrast to an MHC class II-transfected tumor cell line (41), the plasmacytoma used here does not express murine B7-1 and B7-2 in vivo. Using mAbs that do not cross-react with human B7-1, we have clearly demonstrated that host B7-1 molecules are critical for the induction of mature CTLs. Although the rapid rejection of the tumor that expresses high levels of human B7-1 has made it necessary to use a clone that expresses a low level of human B7-1 for the current study, the requirement for host APC is not due to the fact that low level of B7-1 is expressed on the tumor cells, because B7-2 on host APC is involved in CTL responses to a tumor that expresses high level of murine B7-1.

Huang et al. (40) have demonstrated that priming by B7-1-expressing tumor requires presentation of tumor antigen by the host APC. Our current study demonstrates that costimulatory molecules on the host cells are also essential. Thus, both the TCR ligand and the costimulatory molecules on the host APC are essential for the induction of antitumor CTLs. Why then is B7 expression on tumor cells required for the induction of antitumor CTLs? Several interpretations, which are not mutually exclusive, can be advanced to explain this finding. First, as we have reported recently, expression of B7-1 on tumors allows sustained production of MIP1α in tumors, which in turn leads to establishment of lymphoid environment within the tumor (35).

Second, as several groups have reported (25, 36, 37, 40, 42–44), B7-1 on the tumor cells can facilitate the cytolysis of tumor cells by NK cells and T cells, which may allow cross-presentation of tumor antigens by the host APC. Third, it can be argued that maturation of antitumor CTLs may involve multiple steps: one is driven by tumors, and another is driven by the host APC. Although both tumors and host APC can present antigen and costimulator to T cells, these two steps can have different functions in T cell responses. For instance, antigen density on tumors may be higher than that cross-presented on host APC (6, 45). Moreover, host APC present antigen in the context of multiple costimulatory molecules (46–48) in addition to B7.
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Maturation of Cytotoxic T Lymphocytes against a B7-transfected Nonmetastatic Tumor: A Critical Role for Costimulation by B7 on Both Tumor and Host Antigen-presenting Cells

Maja Maric, Pan Zheng, Supria Sarma, et al.


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