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Strong Cytotoxic T Lymphocyte Responses to a Macrophage Inflammatory Protein 1α-expressing Tumor: Linkage between Inflammation and Specific Immunity[1]

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ABSTRACT

Leukocyte infiltration is an important histological sign of resistance to tumors. However, it is unclear whether local inflammatory responses can promote specific antitumor immunity. Here we report that local expression of macrophage inflammatory protein 1α, which results in strong inflammation of leukocytes in tumors, leads to the induction of strong antitumor CTL responses. The induction of CTL effectors requires costimulatory molecules B7-1 and/or B7-2 on host antigen-presenting cells but not on the tumors. These results establish a critical linkage between inflammation and specific immunity.

INTRODUCTION

Adaptive immune responses are usually preceded by innate immunity. It has been suggested that innate immune responses can play a critical role in the initiation of specific immune responses (1–4). One of the important innate immune responses is inflammation, which is manifested in part by infiltration of leukocytes to the site of infection and tissue injuries. Local inflammation recruits multiple subsets of leukocytes into tumors. Many of them, such as macrophages (5, 6), neutrophils (6), and natural killer cells (7), can preferentially lyse tumor cells. Moreover, inflammatory cells produce substances such as nitric oxide and free radicals, which may also contribute to tumor rejection (6, 8). It is unclear, however, whether an inflammatory response can help to initiate specific immunity.

Chemokines play an important role in the initiation of inflammation. MIP1α[3] is a member of the C-C chemokines (9) that are chemotactic for macrophages and lymphocytes (10–12). Mice with a targeted mutation of MIP1α have a severely reduced inflammatory response to influenza virus infection (13). More recently, we have demonstrated that local expression of MIP1α is necessary and sufficient to cause preferential recruitment of CD8 T cells into tumors (14). To address whether inflammation can induce specific antitumor immune responses, we expressed MIP1α in plasmacytoma J558 to induce a strong local inflammation and to investigate the effect of MIP1α on antitumor CTL responses. Here we report that the expression of MIP1α in tumors leads to a strong leukocyte infiltration and induction of specific antitumor CTL responses. The induction of CTLs requires expression of the costimulatory molecules B7-1 and/or B7-2 on the host APCs but not on the tumors. Thus, a local inflammation that recruits both T cells and professional APCs induces antitumor CTLs. These results demonstrate a critical link between inflammation and induction of tumor-specific CTL responses.

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[3] The abbreviations used are: MIP1α, macrophage inflammatory protein 1α; APC, antigen-presenting cell; mAb, monoclonal antibody; TIC, tumor-infiltrating cell.

MATERIALS AND METHODS

Cell Lines, Antibodies, and Experimental Animals. Plasmacytoma J558 cells, which have been transfected with either murine B7-1 (J558-B7) or MIP1α cDNA, have been described (14, 15). Two clones from MIP1α cDNA-transfected plasmacytoma were used for the studies; J558-MIP1α6 produces a high dose of MIP1α (46 ng/10^6 cells/24 h), whereas J558-MIP1αE produces no detectable amount of MIP1α ($<$0.01 ng/10^6 cells/24 h). Macrophage cell line P388D1 (H-2^d), which was obtained from the American Type Culture Collection, was cultured in RPMI 1640 containing 5% FCS and 100 μg/ml each of penicillin and streptomycin.

Antibodies used in this study were: anti-PC.1 mAb 4G6 (16), anti-B7-1 mAb 3A12 (17), 10.16A.1 (18), anti-B7-2 mAb GL-1 (19), anti-macrophage mAb F4/80 (20), anti-dendritic cell mAb N418 (21), and anti-B220 mAb TIB146 (22).

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.

Preparation of TICs. J558-B7, J558-MIP1α6, and J558-MIP1αE 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 using a Ficoll-Hypaque solution. To prepare infiltrating host cells, single-cell suspensions from two to three tumors were pooled and incubated with 1:800 anti-PC.1 mAb ascites for 45 min at 4°C (final concentration of tumor cells was 10^7/ml). The unbound mAbs were removed by two washes with PBS, and goat-anti-rat IgG-coated iron beads (Biosource International, Camarillo, CA) were added at a concentration of 20 beads/cell. The tumor cells, coated with the beads, were removed by a magnet. The remaining nontumor host cells were counted and used for both flow cytometry and cytotoxicity assays.

Quantitation of Inflammatory Responses. The extent of inflammation is determined by the number of host cells in comparison to the total viable cells from the tumors. At different times after tumor injection, tumors were surgically removed, and tumor cells were depleted by a negative selection using anti-PC1 mAb specific for the tumor cells. The viable cells that remained in the solution were counted, and the yield of host cells was expressed as a percentage of viable cells isolated from the tumor.

CTL Assay. The cytotoxicity of TICs was determined by a 6-h CTL assay. Briefly, P388D1 cells pulsed with 10 μg/ml of either P1A antigenic peptide (LPYLGWLVF; Ref. 23) produced by Research Genetics (Huntsville, AL) or a control K^d-binding peptide (KYGVSAQDI) were labeled with ^{51}Cr and incubated with effector cells. In some experiments, J558-B7 and J558-MIP1α were also used as target cells. The amount of released ^{51}Cr was determined by using a gamma counter, and the percentage of specific lysis was calculated as has been described (24).

***In Vivo* Antibody Treatment.** BALB/c mice received s.c. injections of 5×10^6 J558 MIP1α6. These mice also received injections of PBS with anti-B7-1 mAb (3A12) + anti-B7-2 mAb (GL-1) i.p. on days 0, 3, 6, 9, and 12 after tumor injection at a dose of 200 μg/mouse/injection. On days 15 and 17, the tumors were obtained by surgery, and the CTL activity in the tumors was determined *in vitro*.

Tumorigenicity Assay. Syngeneic BALB/c mice were challenged in the left flank with 5×10^6 J558-MIP1αE or J558-MIP1αE cells. The size and incidence of tumors were determined by physical examination.

RESULTS

Local Expression of MIP1α Increases Infiltration of APCs, but It Does Not Alter APC Compositions and B7 Expression. We have transfected the plasmacytoma J558 tumor cell line with cDNA encod-

ing for either MIP1α (14) or the costimulatory molecule B7-1 (15). Three tumor cell lines were chosen for the present studies. J558-B7 expresses costimulatory molecule B7-1 but no detectable MIP1α; J558-MIP1α#6 produces a high dose of MIP1α (46 ng/10^6 cell/24 h), whereas J558-MIP1αE produces no detectable MIP1α ($<$0.01 ng/10^6 cell/24 h). Because our previous study had established that J558-MIP1αE and J558-Neo (J558 cells transfected with vector alone) have similarly low inflammation (14), we used J558-MIP1αE as the negative control for the present study.

Plasmocytoma J558 is of lymphoid origin, which makes it less reliable to quantitate inflammation by histology techniques. However, because J558 tumors express plasma cell marker PC1, we can therefore remove tumor cells using PC1-specific mAb. Because $>$90% of the remaining viable cells express various markers that are expressed

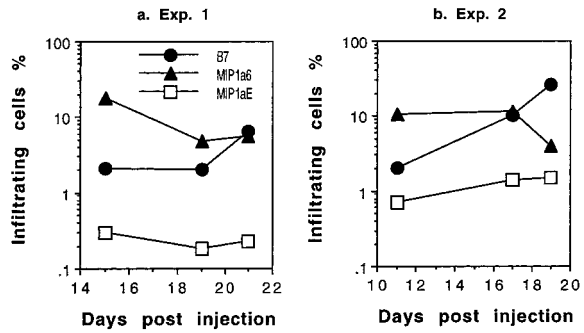

Fig. 1. Local expression of MIP1α increases host-cell infiltration into tumors. *a* and *b*, results from two representative experiments. Syngeneic BALB/c mice received injections of 5×10^6 J558-B7, J558-MIP1α6, or J558-MIP1αE cells. Tumors were surgically removed at different times after inoculation to prepare single-cell suspensions. Tumor cells were removed by negative selection using anti-PC1 mAbs. The viable cells that remained in the solution were counted, and the extent of inflammation was calculated based on the following formula: infiltrating host cells % = (number of cells after selection/number of cells prior to selection) $\times$ 100.

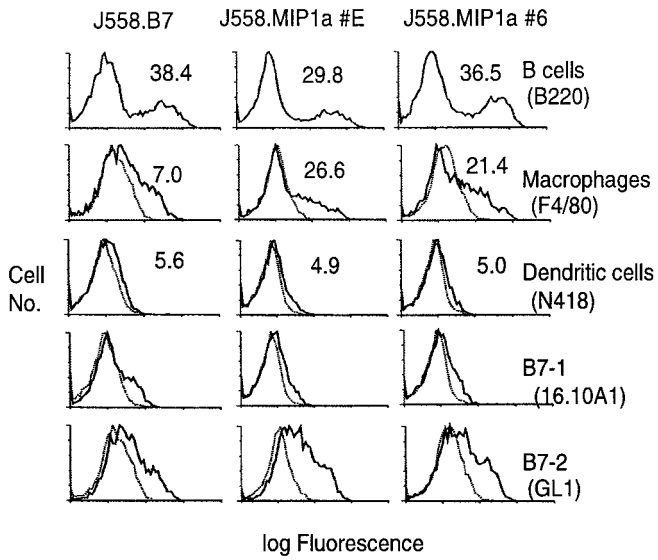

Fig. 2. Expression of MIP1α does not change the APC composition and cell surface expression of costimulatory molecules B7-1 and B7-2. Infiltrating host cells were isolated on day 14 after tumor injection. The amount of B cells was determined by phycoerythrin-labeled anti-B220 mAb; the amounts of macrophages and dendritic cells were determined by unconjugated F4/80 or N418 mAb, followed by FITC-labeled mouse anti-rat immunoglobulin or goat anti-hamster immunoglobulin (mouse/rat immunoglobulin adsorbed). Expression of B7-1 and B7-2 was measured by indirect immunofluorescence using specific mAbs 10.16A.1 and GL-1. *Dotted lines*, distribution of fluorescence in the absence of a primary antibody (negative controls); *solid lines*, those in the presence of primary and second-step reagents. The numbers shown in each panel are the percentages of positive cells after subtraction of those in negative controls.

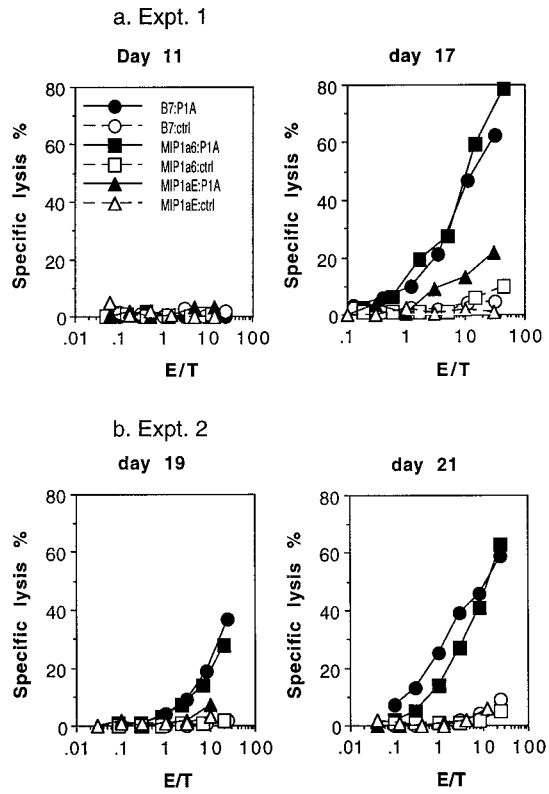

Fig. 3. Local expression of MIP1α enhances anti-P1A CTL response. At given time points after tumor injection, TICs were isolated from pools of two to three tumors expressing B7-1 (*B7*), high dose (*MIP1α6*), or undetectable amounts (*MIP1αE*) of MIP1α, respectively. Without any *in vitro* restimulation, TICs were tested directly on syngeneic target cells (P388D1) pulsed with 10 μg/ml of either P1A antigenic peptide (LPYLGWLVF) or a control K^d-binding peptide (KYGVSAQDI; *ctrl*). Results from two independent experiments, each involving two time points, are presented.

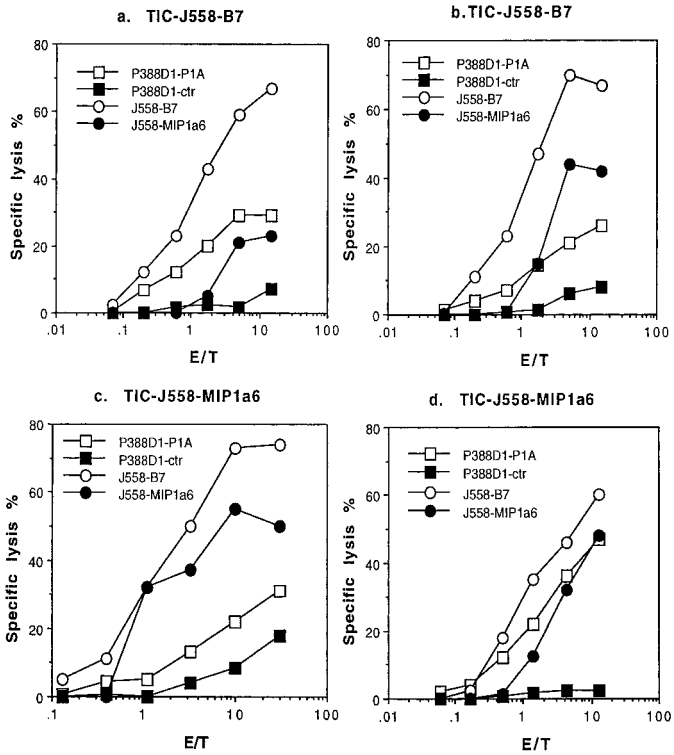

Fig. 4. TICs from J558-MIP1α and J558-B7 are cytotoxic to tumor cells. TICs from two J558-B7 tumors (*a* and *b*) and two J558-MIP1α6 tumors (*c* and *d*) were isolated at day 19 after tumor injection and used as effectors. P1A or control peptide-pulsed P388D1, J558-B7, and J558-MIP1α6 cell lines were used as targets.

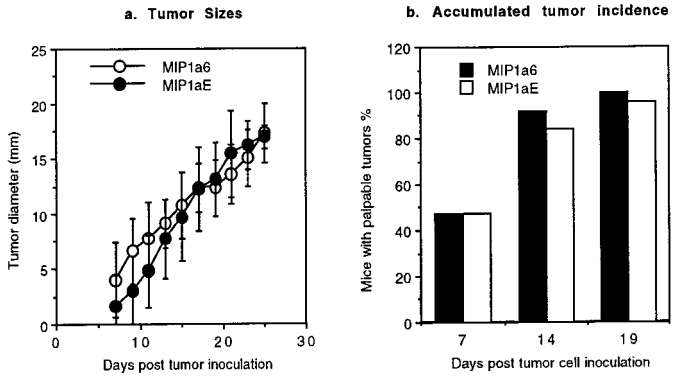

Fig. 5. MIP1α expression does not alter growth kinetics (*a*) or incidences (*b*) of J558 tumors in syngeneic mice. Data presented in *a* were from one experiment involving 8 mice/group, and those in *b* were the accumulated tumor incidence of five independent experiments involving 38 mice/group. *Bars*, SD. All tumors grew progressively until euthanasia became necessary.

on leukocytes but not tumors (data not shown), the yield of cells after negative selection was used to measure the extent of inflammation. The results, which are presented as percentages of viable cells after negative selection, are shown in Fig. 1. J558-MIP1α6, which constitutively secreted MIP1α at high levels, was infiltrated with a large number of host cells that consist of 5–20% of the viable cells in the tumors. On the other hand, J558-MIP1αE, which produces no detectable MIP1α, is infiltrated with significantly fewer host cells. Although J558-B7 is also infiltrated with a large number of host cells, the inflammatory response accelerates with time. This is consistent with our previous finding that MIP1α produced during T-cell response within J558-B7 tumors is responsible for inflammatory responses in J558-B7 tumors (14).

To characterize the APCs infiltrated into tumors, we analyzed infiltrating leukocytes with mAb-specific B cells (B220), macrophages (F4/80), and dendritic cells (N418). As shown in Fig. 2, all major types of APCs, such as macrophages, dendritic cells, and B cells, are represented in the tumor infiltrates. Interestingly, although the total number of host cells is different between the J558-MIP1α6 and J558-MIP1αE tumors (Fig. 1), the relative number of different APCs are similar between the two types of tumors (Fig. 2). Thus, in an *in vivo* setting, MIP1α recruits, directly or indirectly, all types of APCs. Moreover, both the percentage of B7-1/B7-2$^+$ cells and the intensity of B7 on the host cells are essentially identical whether or not MIP1α is expressed constitutively.

MIP1α-mediated Local Inflammation Induces Strong P1A-specific CTLs. We have demonstrated previously that the major tumor antigen in J558 is a P1A peptide presented by L^d (23). In addition, macrophage cell line P388D1 can present the P1A peptide to a P1A-specific CTL line (24). Moreover, because P388D1 was not killed by the TICs in the absence of the P1A peptide (23), it is an ideal tool to reveal P1A-specific CTL responses *in vivo*. To test whether local inflammation initiated by MIP1α is sufficient to induce tumor-specific CTLs, TICs were isolated at various time points and tested for P1A-specific cytotoxicity using the syngeneic P388D1 cells as the targets. Two representative experiments are shown in Fig. 3. Regardless of their tumor sources, TICs recovered on day 11 contained no P1A-specific CTLs. On day 17, strong CTL responses were detected among TICs from J558-B7 and J558-MIP1α6 tumors but not from MIP1αE tumors (Fig. 3*a*). Moreover, in most experiments, CTL responses in MIP1α-expressing tumors can be as long-lived as those from J558-B7 tumors (Fig. 3*b*). MIP1α-expressing tumors have more TICs than the tumor that secreted no detectable MIP1α (Fig. 1). Moreover, TICs from J558-MIP1α tumors are ~30-fold more efficient on a cell-to-cell basis than those isolated from tumors that express no MIP1α (Fig. 3). Thus, local expression of MIP1α promotes inflammation and antitumor CTL responses *in vivo*.

We compared the susceptibility of the J558-B7 and J558-MIP1α6 tumors to the TICs. As shown in Fig. 4, the TICs are cytotoxic for both J558-B7 and J558-MIP1α6 targets (Fig. 4). However, on a cell-to-cell basis, TICs from both J558-MIP1α and J558-B7 are 3–30-fold more efficient in lysing J558-B7 than in lysing J558-MIP1α (Fig. 4). A substantial proportion of the TICs used in the assay are specific for P1A peptides (AA35–43). Because the TIC tumor cells are lysed more efficiently, it is likely that antigens other than P1A are also recognized. Most strikingly, despite the drastic difference in CTL responses, there is no difference between J558-MIP1α6 and MIP1αE tumors in their growth rates in syngeneic BALB/c mice (Fig. 5). These results indicate that the production of CTLs within the tumors is not sufficient to cause tumor rejection.

B7-1 and B7-2 on Host APCs Are Required for CTL Induction. J558 cells do not express costimulatory molecule B7, nor can B7 be induced on tumor cells *in vivo* (25). It may be suggested that P1A-specific CTLs are induced in a B7-independent mechanism. Alternatively, because it is clear that host APCs are essential for the induction of antitumor CTL responses and because a large number of B7$^+$ APCs are present in the tumor (Fig. 2), it is possible that the expression of the costimulatory molecule B7 on the host APCs may be essential. To test whether B7-1 and B7-2 molecules expressed on the host APC play a role in the induction of CTLs, we injected two groups of BALB/c mice with the J558-MIP1α 6 tumor cell line and treated one group with PBS and the other group with anti-B7-1 (3A12) and anti-B7-2 (GL-1) mAbs. The cytotoxic activity of TICs was measured on days 15 and 17 after tumor challenge. In mice treated with PBS alone, a significant CTL response developed by day 15, and the response was substantially stronger on day 17. In mice treated with

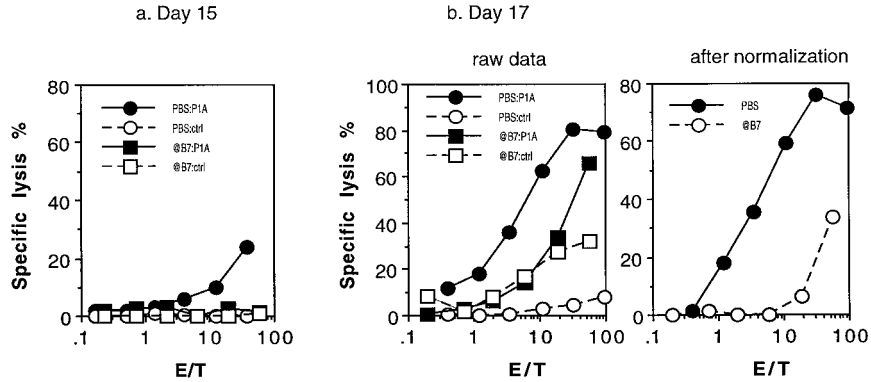

Fig. 6. Requirement for B7 molecules expressed on host APCs. Mice receiving injections of 5×10^6 J558-MIP1α6 tumor cells were treated with either PBS or a mixture of anti-B7-1 and anti-B7-2 mAbs (*@B7*). On days 15 (*a*) and 17 (*b*) after tumor injection, TICs were isolated, and their cytotoxicity was tested using P388D1 pulsed with either P1A or a control K^d-binding peptide. Note that most of the cytotoxicity detected on day 17 in the anti-B7-treated group is not specific for any tumor antigen; after subtracting lysis of control targets, P1A-specific cytotoxicity is minimal in the anti-B7-treated group (*b*, *right*). *ctrl*, control.

anti-B7-1/B7-2 mAbs, however, no CTL activity was detected on day 15. Whereas substantial cytotoxicity was detected in TICs from the anti-B7-treated mice on day 17, the cytolysis was not P1A specific (Fig. 6). Thus, induction of tumor-specific CTLs in MIP1α-expressing tumors requires costimulation by the host APCs. The mechanism for accumulation of nonspecific cytotoxic cells when B7 is blocked is unclear.

DISCUSSION

A fundamental issue in immunology involves the signals that lead to the initiation of immunity. It has been suggested that innate immune responses to microbial infection (1–3) or tissue damage (4) may be crucial signals that switch on specific immunity. A common innate immune response is inflammation. Here we demonstrated that the expression of MIP1α in tumor cells induces a strong CTL response against tumor antigen P1A. To our knowledge, this is the first demonstration that a local expression of chemokine can induce antigen-specific CTLs that are cytotoxic without further *in vitro* restimulation. Our results are in line with a recent study that found that the local expression of tumor necrosis factor in pancreatic islet cells lead to local antigen presentation to CD4 T cells and accelerated development of diabetes (26). Taken together, these results reveal a linkage between inflammation and specific T-cell responses.

We have demonstrated a large amount of MIP1α mRNA in the J558-B7 tumors prior to maturation of antitumor CTL effectors (14, 25). Local accumulation of MIP1α may, in part, be responsible for the induction of antitumor CTLs within the J558-B7 tumors (25). The mechanism by which inflammation promotes specific immunity is not clear. It has been demonstrated that APCs recovered from inflammatory sites have autoantigenic peptides presented by the MHC class II pathway (26). This raises the possibility that inflammation may lead to an accumulation of host APCs that uptake and present antigens in the MHC class I pathway. Several lines of evidence are consistent with this notion: (*a*) we reported that tumor-specific effector CTLs mature within the tumor (25), thus suggesting a requirement for a local antigen presentation; (*b*) this and a previous report from us (25) indicate that the antitumor CTL response requires B7-1/B7-2 on host APCs. This evidence, taken together with the requirement for antigen presentation by MHC class I molecules on hematopoietic host APCs in the induction of antitumor CTLs (27–29), shows that it is most likely that an antigen captured by the host cells is required for the induction of antitumor CTLs.; and (*c*) it has been demonstrated that trafficking of MHC class II molecules can be modified by inflammatory stimuli (30). A critical issue is whether APCs from the tumor milieu can indeed present tumor antigens by way of the MHC class I pathway. Regardless of the mechanism, the direct link between inflammation and induction of specific antitumor CTLs, as demonstrated in the present study, would help to explain the long-standing observation that inflammation indicates better prognosis for malignant tumors (31, 32).

The fact that expression of MIP1α promotes antitumor CTLs but not tumor rejection revealed that, at least for this tumor model, the local production of antitumor effector cells, although necessary (15, 25), is insufficient to cause tumor rejection. At least two hypotheses, which are not necessarily mutually exclusive, can be proposed to explain this paradoxical finding: (*a*) the CTL effector function may have been suppressed by the local factors directly or indirectly linked to MIP1α; and (*b*) in contrast to a large panel of tumors used by several laboratories (33–35), tumor rejection in this and some other (15, 36–38) tumor models also requires expression of B7 on the tumor cells. Inconsistent with this notion is our observation (39) that down-regulation of costimulatory molecule B7-1 on the tumor cells allows tumor evasion of preexisting CTL responses. B7-based immunotherapy for this category of tumors may face an additional obstacle, *i.e.*, the need to express B7-1 on most, if not all, tumor cells *in vivo*. Moreover, the requirement for both B7 and antigens at the effector phase suggest that the "effector" T cells recovered from the tumors are not fully activated. It may be possible to enhance the efficacy of tumor immunotherapy by stimulating the preexisting antitumor CTLs.

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