Intratumoral Expression of MIP-1β Induces Antitumor Responses in a Pre-Established Tumor Model through Chemoattracting T Cells and NK Cells

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Direct intratumoral introduction of therapeutic or regulatory genes is a developing technology with potential application for cancer gene therapy. Macrophage inflammatory protein-1 beta (MIP-1β) is a chemokine which can chemoattract immune cells such as T cells. In the present study, murine colorectal adenocarcinoma CT26 cells were transfected with a recombinant adenovirus (AdhMIP-1β) carrying the human MIP-1β gene. 24 h post-transfection, hMIP-1β levels reached approximately 980 pg/ml in supernatants of 10^6 hMIP-1β-transfected CT26 cells. Moreover, the supernatants exhibited chemotactic activity for $CD8^+$ T cells, $CD4^+$ T cells, NK cells and immature DCs. Intratumoral injection of AdhMIP-1β significantly inhibited tumor growth and prolonged the survival time of tumor-bearing mice. Intratumoral hMIP-1β gene transfer also induced powerful tumor-specific CTL responses *in vivo*. The therapeutic effects of hMIP-1β gene therapy were greatly reduced following *in vivo* depletion of both $CD4^+$ and $CD8^+$ T cells, but were unaffected by depletion of single T cell subsets. Immune cell depletion experiments also revealed that NK cells played an important role in hMIP-1β-induced antitumor responses. These results suggest that intratumoral expression of hMIP-1β has the potential effect to induce host antitumor immunity and may prove to be a useful form of cancer gene therapy. ***Cellular & Molecular Immunology*****. 2004;1(3):199-204.**

Key Words: AdhMIP-1β, transfection, gene therapy, CT26 colorectal adenocarcinoma, antitumor immunity

Introduction

Chemokines are small chemotactic cytokines which can induce migration of leukocytes, activate inflammatory responses, and are implicated in the regulation of tumor development and growth. They can modulate tumor growth *via* regulation of tumor-associated angiogenesis, by activation of host immunological responses or by direct inhibition of tumor cell proliferation (1, 2). Due to their immunoregulatory capacity and pivotal role in induction of antitumor responses, we believe that chemokines hold much promise as a novel mode of treatment for cancer. Indeed, a variety of chemokine-based therapies, including chemokine gene therapies, have been tested for their possible application in cancer treatment. Several chemokines, including MIP-1α, MIP-3α, RANTES and lymphotactin, have been transduced into tumors, triggering antitumor immunity (3-6). In addition, our previous studies have demonstrated that lymphotactin gene-modified dendritic cells act as more potent adjuvants for peptide delivery to induce specific antitumor immunity (7), and also that macrophage-derived chemokine gene transfer results in tumor regression in a murine tumor model through efficient induction of antitumor immunity (8).

Macrophage inflammatory protein-1β (MIP-1β) is an 8 kDa member of the CC chemokine family (1) and appears to be synthesized by a variety of cells, including monocytes, activated T cells and B cells (9, 10). MIP-1β possesses several properties which make it a suitable candidate for cancer gene therapy. MIP-1β is a potent lymphocyte chemoattractant, attracting both T lymphocytes and NK cells (11, 12). Chemotaxis and transendothelial migration assays also indicate that MIP-1β is a potent chemoattractant for immature dendritic cells (DCs), and that upon maturation DCs lose their chemotactic responses to MIP-1β (13, 14). Therefore, expression of MIP-1β within tumors could be used to recruit immature DCs, which could then go on to initiate antitumor T-cell

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Abbreviations: MIP-1β, macrophage inflammatory protein-1β; CTL, cytotoxic T lymphocytes; IL, interleukin; IFN, interferon.

responses. To evaluate the role of this chemokine in tumor immunity *in vivo*, the MIP-1β gene has been delivered into tumors by HVJ cationic liposomes by Miyata T and co-workers (15) and the results showed that the antitumor responses were induced. In the present study, an adenovirus vector encoding human MIP-1β was introduced into established murine tumors. The therapeutic effects were observed and the mechanisms underlying these effects were explored.

Materials and Methods

Animals and cell lines

Male BALB/c mice, 6-8 weeks of age, were purchased from Joint Ventures Sipper BK Experimental Animal Co. (Shanghai, China). Animals were housed under specific pathogen-free condition for all experiments. The human embryonic kidney cell line 293 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). CT26 is a murine colorectal adenocarcinoma cell line derived from BALB/c mice. The 293 cell line was cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Hyclone, Logon, VT), 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (GIBCO BRL, Gaithersburg, MD). CT26 cells were cultured in complete RPMI 1640 medium supplemented as for DMEM.

Adenovirus preparation

Replication-defective recombinant adenovirus AdLacZ encoding β-galactosidase was kindly provided by Dr. Hamada (Department of Molecular Biotherapy Research, Cancer Institute, Tokyo, Japan). Recombinant adenovirus containing the human MIP-1β gene (AdhMIP-1β) was kindly provided by Dr. E Regulier (TRANSGENE SA, Strasbourg, France), this adenovirus was constructed from the human adenovirus serotype 5 using homologous recombination. The expression of the human MIP-1β gene was driven by the CMV promoter and LacZ expression driven by the CAG promoter. Adenoviruses were propagated in 293 cells, and virus titers were determined by plaque assay using 293 cells. The produced recombinant adenovirus were adjusted to a titer of 10^{10} pfu/ml in PBS, aliquoted, and stored at -80 °C.

In vitro transfection of tumor cells

CT26 cells were allowed to grow to confluency in 25 cm^2 tissue culture vessels in complete RPMI 1640 medium. Cells were then rinsed with PBS solution and 2 ml serum free RPMI 1640 containing AdhMIP-1β or AdLacZ (MOI=50) added. 1 h later the medium was removed, and replaced with RPMI 1640.

Assay for hMIP-1β

hMIP-1β gene-transfected CT26 cells were seeded in a 6-well microplate (1×10^6 cells/well), then supernatants collected at every 2 h. Levels of hMIP-1β were determined by ELISA (R&D Systems, MN), performed according to the manufacturer's instructions.

Preparation of $CD4^+$ and $CD8^+$ T lymphocytes, NK cells and immature DCs

Murine splenic mononuclear cells were isolated by Ficoll-Hypaque (density 1.077) gradients. After passing through Nylon wood column (Gibco BRL, Carlsbad, CA), non-adherent cells were collected and further purified using the MiniMACS cell separation system (Miltenyl Biotec, Bergische Aladbach, Germany). $CD4^+$ and $CD8^+$ T lymphocytes were positively selected. The purity, determined by FACS analysis (Becton Dickson, San Jose, CA), was 90% and 93%, respectively. More than 85% of negatively isolated cells were NK cells. Mouse bone marrow-derived DCs were generated as described previously (14). Briefly, bone marrow suspensions from C57BL/6J mice were depleted of red cells using Tris-NH_4Cl and depleted of T cells, B cells, granulocytes, and Ia^+ cells using a cocktail of specific mAbs and rabbit complement. The cocktail consists of anti-CD8 (2.43), anti-CD4 (GK1.5), anti-Ia (B21-2), anti-FcRII (2.4G2), and anti-B220/CD45R (RA3-3A1/6.1) (TIB 210, 207, 229, HB197 and TIB146, ATCC, Rockville, MD). The cells were then cultured in complete medium supplemented with mGM-CSF (3.3 ng/mL) and mIL-4 (5 ng/mL). On the 3rd day of culture, non-adherent cells were removed by gentle pipetting, and the remaining adherent cells were cultured in RPMI complete medium containing mGM-CSF and mIL-4. On the 7th day of culture DC suspensions were harvested for the following experiments.

Chemotaxis of $CD4^+$ T cells, $CD8^+$ T cells, NK cells and immature DCs

To evaluate the chemotactic activity of hMIP-1β protein present in supernatants of CT26 cells infected with AdhMIP-β, chemotaxis of $CD4^+$ T cells, $CD8^+$ T cells, NK cells and immature DCs was analysed. 10^6 $CD4^+$ T cells or $CD8^+$ T cells, NK cells or immature DCs were resuspended in 100 μl of RPMI 1640 containing 0.5% BSA and loaded into the upper well of a transwell chamber (3 μm pore size; Costar, Corning, NY). Different dilutions of supernatants were added to the lower well in a total volume of 600 μl. The chamber was incubated for 4 h at 37 °C in 5% CO_2. The number of cells that migrated to the lower chamber was counted in 5 high power fields. Each experiment was performed in triplicate at least 3 times. The data are presented as mean ± SD.

Treatment of mice with recombinant adenovirus containing hMIP-1β gene

Tumors were established by s.c. injection of CT26 cells. A total of 5×10^5 cells in a volume of 50 μl were injected into the right hind flank of BALB/c syngeneic mice. Five days later, when tumors had reached 4-5 mm (in diameter), the tumors were intratumorally injected with AdhMIP-1β (1×10^9 pfu in 100 μl), control adenovirus AdLacZ (1×10^9 pfu in 100 μl), or PBS (100 μl) three times, at 48 hour intervals. At least 8 mice were in each treatment group. Tumor growth was monitored every 2 days by taking two perpendicular tumor diameter measurements using precision calipers. Tumor area was expressed as the average tumor diameter (mm) ± SD. The survival of tumor-bearing mice was tracked. Mice were euthanized when tumors reached 3 cm in diameter or appeared moribund,

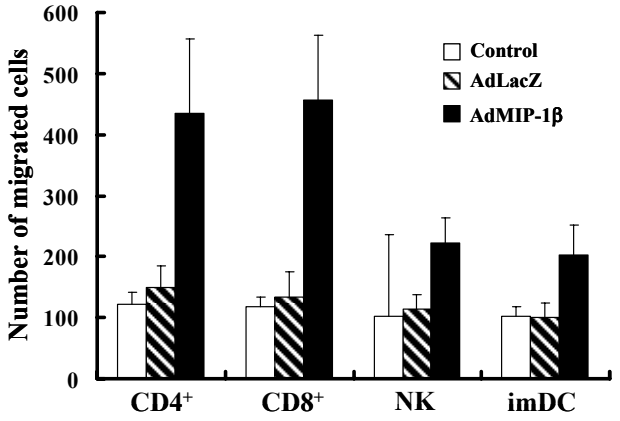

Figure 1. Chemoattractant activity of hMIP-1β gene-transfected CT26 cell supernatants towards immune cells. Magnetic bead-purified $CD4^+$ or $CD8^+$ T cells or NK cells or *in vitro* immature DCs were loaded into the upper well of a transwell chamber, and different dilutions of supernatants of CT26 cells infected for 48 h with AdhMIP-1β, AdLacZ, or uninfected cells added to the lower well. The chamber was incubated for 4 h at 37 ^oC. Migration was expressed as the number of cells that had migrated to the lower well observed in 5 high power fields.

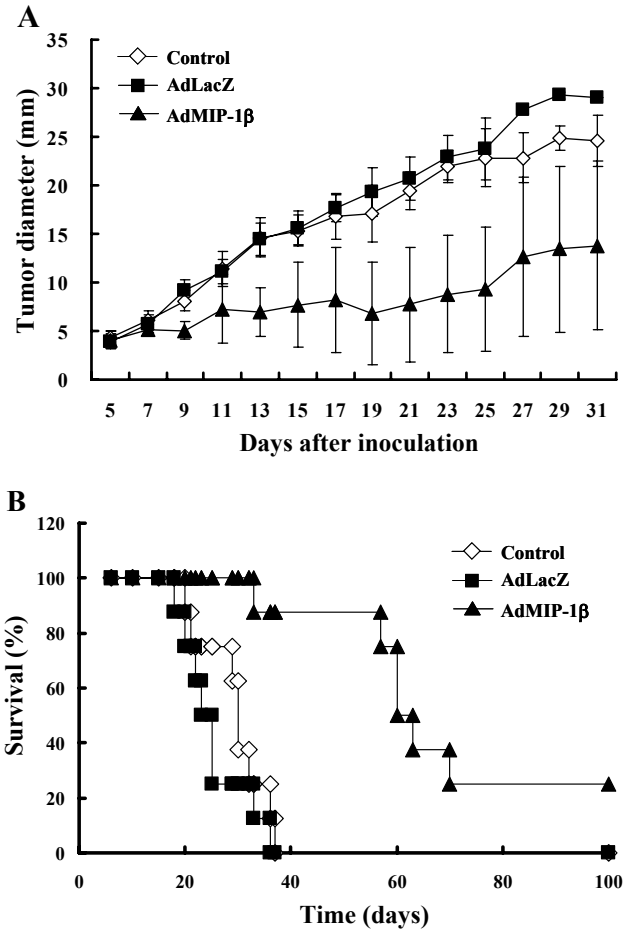

Figure 2. Antitumor effects of intratumoral injection with AdhMIP-1β in a mice with pre-established CT26 colorectal adenocarcinoma. (A) CT26 cells (5×10^5 cells in 50 μl) were inoculated s.c. into BALB/c mice. 5 days later, tumors were intratumorally injected with 1×10^9 pfu AdhMIP-1β, AdLacZ (in 100 μl), or 100 μl PBS. Injections were repeated twice, at 2 day intervals. Tumor size was monitored every 2nd day and is expressed as the average tumor diameter ± SD. (B) Survival time of tumor-bearing mice treated with AdhMIP-1β, AdLacZ or PBS intratumoral injection. Mice were killed when tumor reached 3 cm in diameter or appeared moribund.

and dates of death recorded for survival studies. 10 days after the final intratumoral injection tumor nodules were isolated, fixed in 10% formalin, embedded in paraffin, and sliced for HE staining. Data are representative of three experiments performed.

Cytotoxicity assay of tumor-specific cytotoxic T lymphocytes (CTL)

Splenocytes (10^6 cells/ml) were isolated 10 days after the final adenovirus intratumoral injection and restimulated with irradiated (80Gy) CT26 cells (1×10^7 cells/ml). The mixed cells were cultured in RPMI 1640 (10% FCS) containing 50 U/ml IL-2 for 6 days, then cells harvested as CTL. CTL cytotoxicity was measured *in vitro* using the lactate dehydrogenase (LDH) release assay according to the manual provided with the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega). Briefly, CT26 cells were used as target cells cocultured with effector cells (CTL) at various ratios for 6 h in 96-well round-bottomed plates in RPMI 1640. Spontaneous release of effector and target cells was controlled by separate incubation of the respective cell populations. The percentage cytotoxicity was calculated according to the following formula: Cytotoxicity % = [(E-Se-St) / (Mt-St)] × 100. Where E stands for the LDH release by effector-target cocultured; Se, the spontaneous release by effector cells alone; St, the spontaneous release by target cells alone; and Mt, the maximal release by target cells.

In vivo depletion of immune cell subsets

Monoclonal antibodies anti-CD4 (GK1.5, TIB 207; ATCC), anti-CD8 (2.43, TIB 210; ATCC), and anti-NK1.1 (PK136, HB 191; ATCC) were used to deplete $CD4^+$ T cells, $CD8^+$ T cells and NK cells. Antibodies (100 μg per injection) in 100 μl PBS were injected intraperitoneally into each mouse on days -4 and -1 preceeding inoculation with tumors. An additional three antibody injections were administered at 3-day intervals following inoculation with CT26 cells.. Normal rat IgG (Sigma) was given as negative control. Depletion of T cell subsets and NK cells was monitored by flow cytometry, which showed greater than 90% specific depletion from splenocytes (data not shown).

Statistical analysis

The data are presented as mean ± SE. Statistical analysis was performed using student's *t*-test, with statistical significance determined at $p<0.05$. Survival estimates were determined using the method of Kaplan and Meier.

Results

hMIP-1β expression and chemoattractive activity arising from AdhMIP-1β transfection of CT26 cells

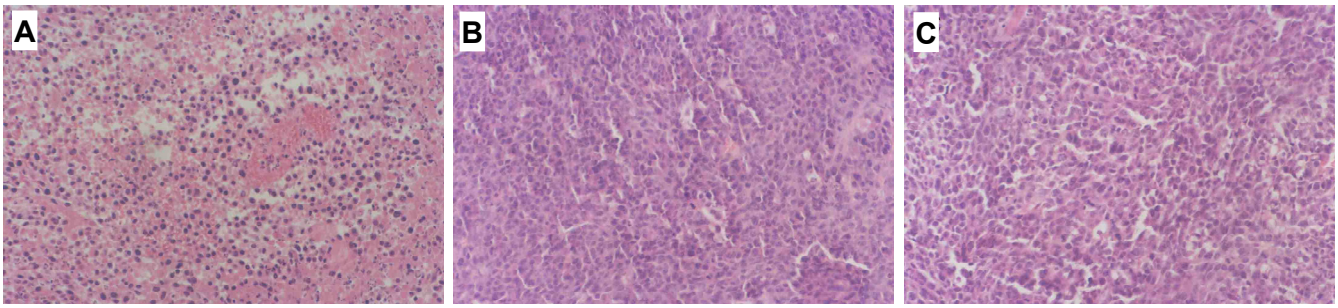

Figure 3. Pathological analysis of tumor tissues treated with intratumoral injection of AdhMIP-1β. Established CT26 tumors were subjected to a regine of intratumoral injections with either AdhMIP-1β (A), AdLacZ (B) or PBS (C), 10 days after the final injection tumor nodules were prepared for histological analysis. Images were captured under 200 magnification.

Supernatants of transfected CT26 cells were harvested and hMIP-1β production determined by ELISA. Approximately 76 pg/ml hMIP-1β could be detected 4 h after AdhMIP-1β transfection, with hMIP-1β expression reaching it's highest level (about 980 pg/ml) at 24 h. hMIP-1β was not detected in the supernatants of untransfected or AdLacZ transfected CT26 cells. The hMIP-1β gene could thus be efficiently transduced into CT26 cells, resulting in secretion of hMIP-1β protein.

To verify if hMIP-1β present in supernatants was functional, it's chemoattractive activity towards different kinds of immune cells was assessed by quantifying $CD4^+$ and $CD8^+$ T cells and NK cells and imDCs migration across 3 μm pore polycarbonate membranes in a transwell cell culture chamber. As shown in Figure 1, supernatants from hMIP-1β gene-transfected CT26 cells displayed marked chemotactic activity to $CD4^+$ T cells, $CD8^+$ T cells, NK cells and imDCs compared with LacZ gene-transfected CT26 cells and control (p<0.01).

Tumor growth after intratumoral human MIP-1β gene transfer

The antitumor effects of AdhMIP-1β administration were investigated in a murine model of pre-established CT26 colorectal adenocarcinoma. As shown in Figure 2A, treatment of tumor-bearing mice by intratumoral injection of AdhMIP-1β resulted in significant regression of pre-established tumors, as compared with administration of AdLacZ or PBS (p<0.01). Moreover, as shown in Figure 2B, survival of tumor-bearing mice in the group administered with AdhMIP-1β was significantly longer than that of mice treated with AdLacZ or PBS (p<0.01), indicating that potent antitumor effects might be induced by intratumoral hMIP-1β gene transfer.

Appearance of tumor tissue after adminstration of intratumoral human MIP-1β adenovirus injection

We also observed tumor tissue pathology following intratumoral administration of AdhMIP-1β, AdLacZ or PBS. As shown in Figure 3, overt necrosis and lymphocyte infiltration were observed in tumor tissues treated with hMIP-1β-adenovirus, but not in those treated with LacZ adenovirus or PBS. These data indicated that intratumoral injection of AdhMIP-1β was able to recruit immune cells into tumor tissues, with these cells most likely exerting a cytotoxic effect on tumor cells, leading to necrosis.

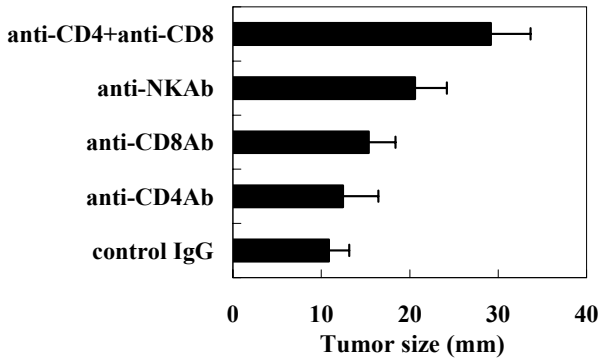

Figure 4. Effector cells involved in antitumor response brought about by MIP-1β gene therapy. *In vivo* depletion analysis with anti-CD4, anti-CD8 or anti-NK mAbs was performed as described in Materials and Methods.

Effector cells involved in antitumor responses elicited by intratumoral administration of AdhMIP-1β

To further characterize the effector cells involved in the antitumor effects of hMIP-1β gene therapy, anti-CD4 mAb, anti-CD8 mAb or anti-NK mAb mediated depletion was performed to elucidate the roles of these subsets. Specific depletion of either $CD4^+$ or $CD8^+$ T cells resulted in a minor decrease in the antitumor response. However depletion of both $CD4^+$ and $CD8^+$ T cells resulted in lethal tumor progression, despite intratumoral administration of AdhMIP-1β. Depletion of NK cells also allowed the progression of tumors treated with AdhMIP-1β (Figure 4). The results indicated that the tumor growth inhibition resulting from AdhMIP-1β intratumoral administration was both $CD4^+$ and $CD8^+$ T cell-dependent, and that NK cells might also play a partial role.

Tumor-specific CTL activity induced by AdhMIP-1β gene transfer

10 days after AdhMIP-1β administration, splenocytes were isolated and restimulated *in vitro* with irradiated CT26 cells to induce propagation of CTL. 6 days after restimulation,

CTL were harvested and used in cytotoxic assays at effector/target ratios (E:T) of 12.5:1, 25:1 and 50:1. As shown in Figure 5, splenocytes obtained from AdhMIP-1β treated mice gererated significant cytotoxic activity against CT26 cells, as compared with splenocytes obtained from AdLacZ or PBS treated groups ($p<0.01$). Our data suggested that transfection with AdhMIP-1β could indeed elicit tumor-specific CTL activity in tumor-bearing mice.

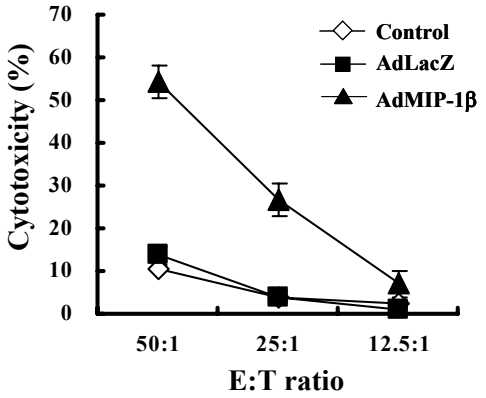

Figure 5. Cytotoxicity of tumor-specific CTL induced by intratumoral injection of AdhMIP-1β. Splenocytes were isolated 10 days after the last AdhMIP-1β, AdLacZ or PBS intratumoral injection 1×10^7 cells/ml restimulated with 1×10^6 cells/ml irradiated (80Gy) CT26 cells to propagate for CTL. 6 days after restimulation, CTL were harvested their ability to lyse CT26 tumor cells at effector/target ratios (E:T) of 12.5:1, 25:1 and 50:1 analyzed by LDH release assay.

Discussion

Gene therapy offers new opportunities for cancer treatment and prevention through the use of targeted, therapeutic, or regulatory genes that can identify, disable, and destroy malignant cells (2). The targeting of molecular events involved in the development and maintenance of malignancies, providing a molecular approach to eradicate cancer cells or to protect normal cells from the toxicity of conventional therapies (16). To date, hundreds of clinical protocols involving cancer gene therapy have been undertaken, and many of these are cytokine gene therapies. Cytokines have diverse functions involving modulation of immune responses and have been employed as biological drugs to treat cancer. Chemokines are a superfamily of small proteins that play a central role in immune response and inflammation by chemoattracting leukocytes (17-19). *In vitro* and *in vivo* data highlight the role of chemokines in the regulation of immune cell emigration from the vascular compartment to inflammatory or tumor sites (20). A variety of chemokines have been demonstrated to possess antitumor activity. MCP-1 reduces *in vivo* growth of tumor cells and increases infiltration of macrophages/monocytes to tumor sites (21, 22), while RANTES and lymphotactin can inhibit tumor formation and generates tumor immunity (3, 4). MIP-1α expression in adenocarcinoma cells leads to reduced tumor formation and increase infiltration of macrophages and neutrophils (5). Adenovirus-mediated gene transfer of MIP-3α to tumors induces local accumulation of DCs and inhibits growth of preexisting tumors (6).

In the present study, we found that supernatants of MIP-1β gene-transfected CT26 colorectal adenocarcinoma cells had potent chemotactic activity to $CD4^+$ T cells, $CD8^+$ T cells, NK cells and immature DCs. When AdhMIP-1β was injected into tumors, lymphocytes were recruited to tumor sites. The antitumor activity was dependent on both $CD4^+$ and $CD8^+$ T cells, being completely reversed upon depletion of both $CD4^+$ and $CD8^+$ T cell subsets. NK cells also appeared to play an important role in tumor rejection. Our results demonstrated that administration of AdhMIP-1β elicited a striking increase in tumor-specific CTL activity. Our study revealed a novel role of hMIP-1β for generation of antitumor immunity and suggested that this is a feasible, effective approach to cancer gene therapy.

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