The Immunization Site of Cytokine-Secreting Tumor Cell Vaccines Influences the Trafficking of Tumor-Specific T Lymphocytes and Antitumor Efficacy against Regional Tumors[1]

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Tumor cells engineered to secrete cytokines, referred to as tumor cell vaccines, can often generate systemic antitumor immunity and, in many cases, cause tumor regression. We compared the efficacy of s.c. immunization or intrahepatic immunization of GM-CSF-expressing tumor cell vaccines on the growth of s.c. or orthotopic liver tumors. A chemically transformed hepatic epithelial cell line, GP7TB, derived from Fischer 344 rats, was used to generate tumor models and tumor cell vaccines. Our results demonstrated that two s.c. injections of an irradiated tumor cell vaccine significantly controlled the growth of s.c. tumors, but was completely ineffective against orthotopic liver tumors. Effector cell infiltration in liver tumors was markedly reduced compared with s.c. tumors. Enhanced apoptosis of some effector cells was observed in the liver tumors compared with the s.c. tumors. Furthermore, the T cells induced by s.c. immunization preferentially migrated to s.c. tumor sites, as demonstrated by adoptive transfer experiments. In contrast, intrahepatic immunization, using parental tumor cells admixed with adenoviruses carrying the GM-CSF gene, yielded significantly better therapeutic effects on the liver tumors than on the s.c. tumors. Adoptive transfer experiments further confirmed that the T cells induced by liver immunization preferentially migrated to the liver tumor sites. Our results demonstrate that distinct T cell populations are induced by different immunization routes. Thus, the homing behavior of T cells depends on the route of immunization and is an important factor determining the efficacy of immunotherapy for regional tumors. ***The Journal of Immunology,*** **2004, 173: 6025–6032.**

Numerous studies have demonstrated that tumor cells engineered to secrete cytokines, here referred to as tumor cell vaccines, can induce significant antitumor immunity and even cause tumor rejection (1). Among the various cytokines that have been evaluated to stimulate immune responses against tumors, GM-CSF is one of the most potent, and its use in tumor cell vaccines has achieved some success in animal tumor models (2–5). The key role of GM-CSF as an immunomodulator is its ability to recruit and activate functional APCs (6, 7), such as dendritic cells (DCs),[4] the most potent activators of T cells.

Immunomodulatory genes have been used to treat hepatocellular carcinoma in animals. However, previous animal studies demonstrating the efficacy of immunotherapy against hepatocellular carcinoma have mostly examined extrahepatic sites for tumor formation, particularly at s.c. sites (8–10). Indeed, most published reports have evaluated tumor immunotherapy against s.c. xenografts due to the ease of inoculating and monitoring tumor growth at this site. However, s.c. tumors may differ immunologically from those in orthotopic tissues. Subcutaneous sites contain relatively high numbers of APCs, they are frequented by circulating lymphocytes, and they are well provisioned with lymphatic and blood vessels. Thus, s.c. tumors may exhibit high inherent immunogenicity (11, 12). In contrast, the liver, for example, is speculated to promote immunological tolerance to foreign Ags (13, 14) and has been demonstrated to attract activated $CD8^+$ T cells undergoing Ag-induced apoptosis (15, 16). Furthermore, it has been demonstrated that effector and memory T cells exhibit tissue-specific trafficking, achieved by regulated expression of particular homing receptors on their surface (including adhesion and chemokine receptors) and by regulation of their counterreceptors on local endothelial cells in the target tissues (17–19). The lymph node (LN) microenvironment determines the fate of effectors and memory T cells during their activation (20). Thus, it is conceivable that the effector cells elicited by a particular immunization route may differentially home to tumors present at different sites in the body. The successful generation of antitumor immunity against s.c. tumors may not necessarily indicate efficacy against orthotopic tumors. Therefore, orthotopic tumor models are preferred for analyzing the effects of immunotherapy in a realistic microenvironment.

We established an orthotopic liver tumor model using a transformed rat hepatic epithelial cell line, GP7TB. For comparison, an s.c. tumor model was established as well. The therapeutic effects of different immunization routes on the orthotopic liver tumors and the s.c. tumors were evaluated. It was found that s.c. immunization with GM-CSF-secreting tumor cell vaccines resulted in significant therapeutic effects on the s.c. tumors, but not on the orthotopic liver tumors. On the contrary, intrahepatic immunization with tumor cells admixed with adenoviruses carrying the GM-CSF gene

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[4] Abbreviations used in this paper: DC, dendritic cell; LN, lymph node; Ad-GM-CSF, adenoviral vector containing a GM-CSF cDNA; Ad-GFP, adenoviral vector containing a GFP gene; CLA, cutaneous lymphocyte-associated Ag.

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had better efficacy on the orthotopic liver tumors compared with the s.c. tumors. Mechanistic studies provided evidence suggesting that the site of immunization markedly influenced the homing capability of tumor-specific T lymphocytes and their ability to control the outgrowth of tumors at different sites in the body.

Materials and Methods

Cell lines and vectors

GP7TB is a cell line derived from chemically transformed hepatic epithelial cells in a Fischer 344 rat (21). RT-2 is a glioma cell line derived from an avian sarcoma virus-induced brain tumor in the Fischer 344 rat (22). A retroviral vector containing a GM-CSF cDNA was used to transduce GP7TB cells following previously described procedures (23). The stable GM-CSF-transduced GP7TB cells (designated as GM/GP7TB) and the control vector-transduced GP7TB cells (designated as S2/GP7TB) were maintained in a selective medium containing G418 (0.8 mg/ml; Sigma-Aldrich, St. Louis, MO). An adenoviral vector containing a GM-CSF cDNA (Ad-GM-CSF) or a GFP gene (Ad-GFP) was constructed as described previously (24).

Generation of orthotopic liver tumors, spleen tumors, or s.c. tumors

Male Fischer 344 rats aged ~7–8 wk, obtained from the National Taiwan University Animal Center, were used in these experiments. The experimental protocols were performed under the National Taiwan University Guidelines for Animal Care. Orthotopic liver or spleen tumors were generated by the insertion method. Briefly, 1×10^7 GP7TB tumor cells suspended in 0.1 ml of HBSS were s.c. inoculated in the posterior flanks of the rats. The tumors that had formed after 1 mo were excised and cut into 1-mm^3 cubes. New naive animals were anesthetized with 90 mg/kg ketamine hydrochloride, their abdominal cavities were opened to expose the liver or the spleen, and a single tumor fragment was inserted into the left liver lobe or the spleen, respectively, using forceps. The incision was subsequently sutured. The animals were randomized and used for treatment experiments. The s.c. tumors were generated in a similar way, except that tumor fragments were inserted in the posterior flank.

When adenoviral vectors or adoptive T cell therapy was used as the treatment strategy, orthotopic liver tumors were generated by directly injecting GP7TB cells into the liver using a syringe (Terumo, Elkton, MD). Notably, the needle hole was sealed with an electric coagulator immediately after the withdrawal of the needle to avoid leakage of the injected fluid. The incision was subsequently sutured. When direct injection of tumor cells was used to generate the liver tumors, the same method was used to generate the s.c. tumors for parallel comparison. The spleen tumors were all generated by tumor fragment insertion to avoid metastasis to the liver.

Tumor treatment and measurement

This study used two strategies to treat liver, s.c., or spleen tumors. The first strategy involved s.c. immunization of the tumor-bearing animals with irradiated tumor cell vaccines. Animals with tumor fragments inoculated in the respective sites were s.c. treated with two doses (3×10^7 cells/dose) of irradiated GM/GP7TB or S2/GP7TB (as a control) tumor cell vaccine on days 1 and 4 after tumor fragment implantation. The vaccination site was opposite to the tumor site if s.c. tumors were treated. On day 42, hepatic and splenic tumor growth was evaluated by surgically opening the abdomens of animals and measuring the tumor size using calipers. Subcutaneous tumors were directly measured using calipers.

The second treatment strategy involved intrahepatic immunization of the tumor-bearing animals with adenoviral vectors. On day 0, animals were inoculated with tumor cells (3×10^6) in one liver lobe or in the subcutis, or with tumor fragments in the spleen. Three days later, the animals were injected with 3×10^6 GP7TB tumor cells admixed with PBS, 3×10^9 PFU Ad-GM-CSF, or 3×10^9 PFU Ad-GFP (in 100 μl) in another liver lobe. The sizes of liver tumors, s.c. tumors, or spleen tumors were measured on day 28 after tumor implantation.

Tumor volumes were calculated using the following formula: volume = $0.52 \times A \times B^2$, where A and B are the longest and the shortest diameter of the tumors, respectively.

Isolation of lymphocytes from tumors and spleens for flow cytometry

To isolate liver tumor infiltrates, the liver was first perfused by injecting 200 ml of HBSS buffer into the hepatic artery. Liver tumors were then resected. Tumor-infiltrating lymphocytes were isolated as previously described (25). Briefly, resected liver or s.c. tumors were cut into small pieces using a razor blade. The tissue fragments were incubated for 15 min at 37°C in HBSS solution (1 g/10 ml) containing collagenase type I (0.05 mg/ml), collagenase type IV (0.05 mg/ml), hyaluronidase (0.025 mg/ml), and soybean trypsin inhibitor (1 mg/ml) (all from Sigma-Aldrich) and DNase (0.01 mg/ml; Roche Applied Science, Mannheim, Germany). Cells were recovered by centrifugation and suspended again in a fresh aliquot of the HBSS digestion solution for 15 min at 37°C. Undigested material was removed on a 40-μm mesh sieve, and the liberated cells were recovered and washed with RPMI 1640 medium. They were further separated on a Ficoll-Paque gradient to remove dead cells. Single cell suspensions of splenocytes were made by pressing the tissue through a 40-μm mesh sieve and washing twice with RPMI 1640 medium. The cells obtained were used for cytometric analysis or for in vitro activation (see below).

Flow cytometry

Cells were stained with directly conjugated Abs, which were obtained from BD Pharmingen (San Diego, CA) unless indicated otherwise. These were FITC-conjugated mouse anti-rat CD4 (clone OX-35; Serotec, Oxford, U.K.), CD8a (clone OX-8; Serotec), macrophage CD11b/c (clone OX-42), and NK CD161a (clone 10/78); mouse anti-human CCR4 (clone 1G1.1); rat anti-human cutaneous lymphocyte-associated Ag (CLA; clone HECA-452); and rat anti-mouse lymphocyte Peyer's patch adhesion molecule-1 (integrin $\alpha_4\beta_7$ complex; clone DATK32). For apoptosis analysis, tumor-infiltrating lymphocytes were first stained with PE-conjugated mouse anti-rat CD4, CD8, macrophage, or NK Ab, followed by staining with annexin V (Annexin V:FITC apoptosis detection kit; BD Pharmingen), according to the manufacturer's instructions. Flow cytometry acquisition was performed on a FACScan apparatus (BD Biosciences, Mountain View, CA). Analysis was performed using the CellQuest program (BD Biosciences).

Activation of tumor-specific T cells for adoptive T cell therapy or in vivo T cell migration assays

Splenocytes were isolated on day 28 after tumor implantation from the animals that received s.c. or intrahepatic immunizations. In vitro stimulation was conducted by culturing 5×10^6 splenocytes with 1×10^5 irradiated GP7TB cells per well in 24-well plates for 5 days at 37°C in the presence of recombinant human IL-2 (10 U/ml). The activated tumor-specific T cells were used for adoptive T cell therapy. Animals were inoculated with GP7TB tumor cells (5×10^6) in the liver or in the subcutis. Three days later, specified numbers of activated T cells were intratumorally injected. Tumor sizes were measured on day 28 post-tumor implantation. For in vivo T cell migration assays, the T cells after in vitro activation were further fractionated on a Ficoll-Paque gradient to remove dead cells. The recovered cells were incubated for 10 min at 37°C in RPMI 1640 medium (1×10^7 cells/ml) containing 5 μM CFSE (Molecular Probes, Eugene, OR). A total of 1×10^7 CFSE-labeled T cells was injected into the tail veins of animals bearing a GP7TB or an RT-2 tumor either in the liver or in the subcutis. Rats were killed 24 h after injection. Total lymphocytes were collected from both tumor sites and some organs. The number of $CFSE^+$ cells in each organ was quantified by flow cytometry.

Statistical analysis

Statistical comparison of the tumor sizes among different groups was performed using one-way ANOVA, whereas comparison of the levels of cellular infiltrates or $CFSE^+$ cells between two tumor sites was performed using nonparametric statistical analysis (Wilcoxon test). Differences were considered statistically significant when $p < 0.05$.

Results

Irradiated tumor cell vaccines administered s.c. could control s.c. tumor but not liver or spleen tumor outgrowth

Numerous studies have demonstrated that cytokine-secreting tumor cell vaccines administered s.c. could allow efficient regression of many different s.c. tumor models (2–5). We investigated whether a similar immunization strategy was effective on an orthotopic liver tumor model. A stable, GM-CSF-expressing GP7TB clone, GM/GP7TB, was obtained by infecting GP7TB cells with a retroviral vector containing the mouse GM-CSF cDNA and was used as a tumor cell vaccine. The control vector-transduced GP7TB clone, S2/GP7TB, served as a control.

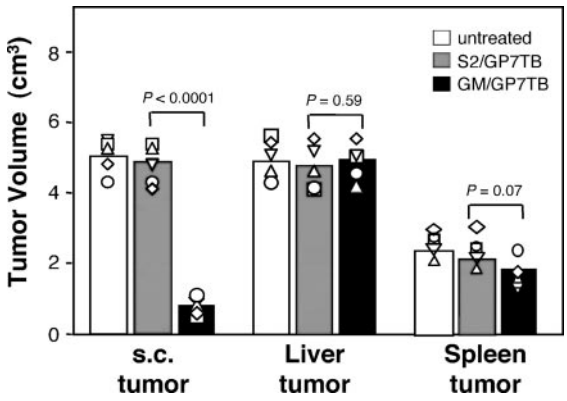

FIGURE 1. Better efficacy of s.c. immunization on s.c. tumors compared with orthotopic liver or spleen tumors. Rats bearing GP7TB tumor fragments in the subcutis, the liver, or the spleen were treated s.c. with irradiated S2/GP7TB or GM/GP7TB tumor cell vaccines as described in *Materials and Methods*. Tumor volume was measured on day 42 after tumor implantation. Each group comprised five rats. The bars represent the mean tumor volume of each group. The volumes of the S2/GP7TB- and the GM/GP7TB-treated s.c. tumors were significantly different ($p < 0.0001$, one-way ANOVA).

Animals were implanted with viable tumor fragments in either their livers or their left flanks on day 0. Irradiated tumor cell vaccines, GM/GP7TB or S2/GP7TB, were then injected in the right flanks of the animals on days 1 and 4 after tumor implantation, and tumor sizes were measured on day 42. Fig. 1 illustrates that s.c. immunization of two doses of the irradiated GM/GP7TB vaccine significantly controlled the growth of s.c. tumors compared with treatment with the S2/GP7TB vaccine or without treatment. In contrast, s.c. injection of the GM/GP7TB or S2/GP7TB vaccine did not suppress the outgrowth of orthotopic liver tumors. To understand whether the effectiveness of s.c. immunization was only specific to s.c. tumors, we further implanted tumors in the spleen and treated the animals by the same strategy. The data shown in Fig. 1 reveal that s.c. immunization with GM/GP7TB was also ineffective against the spleen tumors. Together, these results indicate that even though the s.c. administered GM/GP7TB cell vaccine elicited significant levels of tumor-specific immunity in the animals, as evidenced by its ability to cause regression of the s.c. tumors and to induce CTL activity in the animals (26), it could not control the outgrowth of orthotopic liver or spleen tumors.

Significantly lower levels of cellular infiltrates were present at the liver tumor sites as compared with the s.c. tumor sites

The markedly different therapeutic effects of s.c. immunization of tumor cell vaccines on the s.c. and liver tumors prompted us to explore the cellular mechanisms underlying the phenomenon. Cellular infiltrates (CD8, CD4, macrophages, and NK cells) at both tumor sites were systematically analyzed by flow cytometry. As illustrated in Fig. 2, low numbers of effector cells were observed at the s.c. tumor sites of the untreated animals or the animals treated with the S2/GP7TB vaccine, whereas at the s.c. tumor sites of the animals treated with the GM/GP7TB cell vaccine, the cellular infiltrates started appearing on day 2 after tumor implantation (i.e., 1 day after vaccine treatment), and they quickly accumulated with time up to day 7. Conversely, abundant levels of infiltrates were observed on day 2 at the liver tumor sites of all the groups, irrespective of whether the animals received vaccine. The infiltrating cells in liver tumors, however, rapidly decreased with time. In the GM/GP7TB-treated group, the number of infiltrating cells in liver tumors decreased to lower levels than were measured at the s.c. tumor sites by day 4. These results indicate that the increasing levels of cellular infiltrates at the s.c. tumor sites resulted from immunization with GM/GP7TB cells, but also raise an interesting

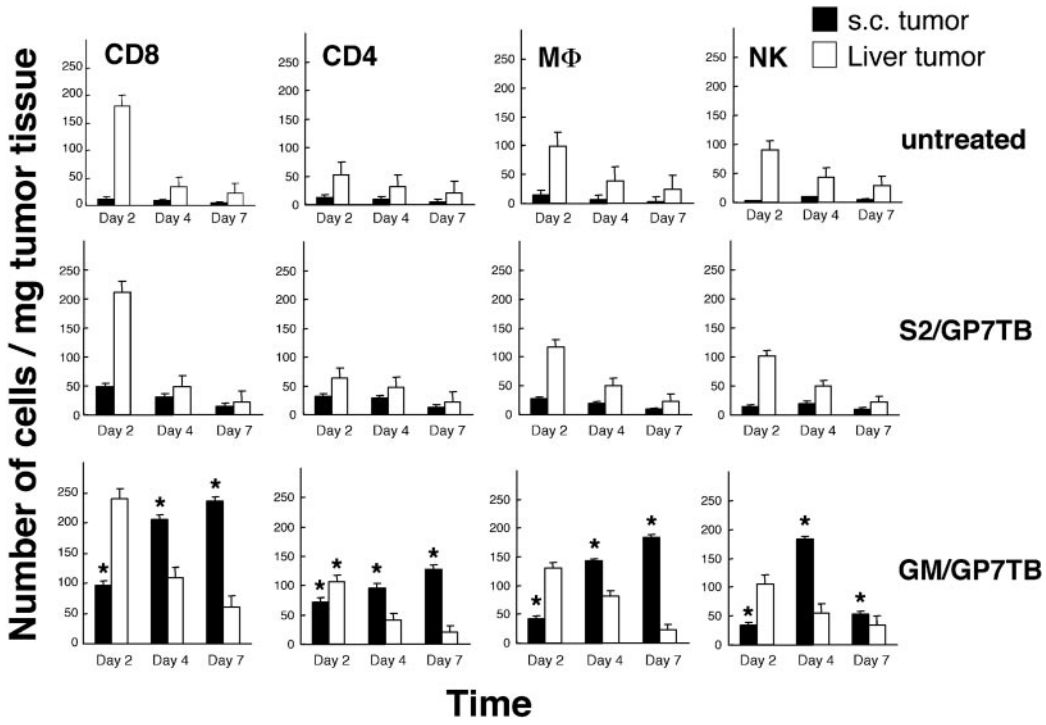

FIGURE 2. Cellular infiltrates at the s.c. or the liver tumor sites. Mononuclear cells were isolated from the tumor sites of the untreated animals or the animals treated s.c. with S2/GP7TB or GM/GP7TB vaccines on days 2, 4, and 7 after tumor implantation. They were stained with Abs against CD4, CD8, macrophages, or NK cells. The bars represent mean cell numbers ± SD of five rats at each time point. *, $p < 0.05$, GM/GP7TB-treated group vs S2/GP7TB-treated group of the tumors implanted at the same site at the corresponding time point (Wilcoxon test).

question as to the origin of the infiltrates at the liver tumor sites. Because abundant cellular infiltrates were also observed in the day 2 liver tumors of the untreated and the S2/GP7TB-treated animals, and the infiltrate levels in the liver tumors of the GM/GP7TB-treated animals were barely or not significantly higher than those in the liver tumors of the S2/GP7TB-treated groups at all three time points (Fig. 2). We speculate that these infiltrates were caused by inflammation due to tumor implantation in the liver rather than from immunization with the GM/GP7TB tumor cell vaccine. This interpretation suggests that s.c. immunization with two doses of the irradiated GM/GP7TB vaccine does not induce significant levels of effector cells to enter into the liver tumors.

Enhanced apoptosis of some effector cells was observed in the liver tumor regions

Two possibilities may explain the low levels of vaccine-induced infiltrates present in the liver tumors of the animals treated s.c. with GM/GP7TB vaccine. First, the effector cells induced by the vaccine may undergo greater apoptosis in the liver tumors than in the s.c. tumors. Second, the effector cells induced by s.c. vaccination may preferentially migrate to the s.c. tumor sites.

We first investigated whether greater apoptosis of infiltrating cells was observed in the liver tumors compared with the s.c. tumors. Cellular infiltrates from both tumor sites were isolated and stained with annexin V. Fig. 3*A* shows that significantly higher levels of apoptotic $CD4^+$ T cells, macrophages, and NK cells were observed in the liver tumor regions compared with the s.c. tumor regions, particularly on day 4. Yet, $CD8^+$ T cells at liver tumor sites did not undergo greater apoptosis than did those at s.c. tumor sites.

We also investigated whether the liver microenvironment could inhibit the antitumor activity of activated CTLs that were directly injected in the liver tumors to eliminate differential migration effects. Tumor-specific T cells were isolated from animals treated s.c. with the GM/GP7TB vaccine, in vitro activated with irradiated GP7TB cells, and then adoptively transferred to animals bearing orthotopic liver tumors or s.c. tumors by direct intratumoral injection. Fig. 3*B* shows that the adoptively transferred T cells caused regression of both s.c. and liver tumors in a dose-dependent manner, and at equivalent efficiencies.

Taken together, our data show that enhanced apoptosis of some effector cells does occur in the liver environment, but that the liver does not inhibit the antitumor activity of activated T cells once they reach the liver. Our results suggest that apoptosis in the liver could partly, but certainly inadequately, account for the lower levels of cellular infiltrate in the liver tumors compared with the s.c. tumors, and they further suggest that T cell trafficking may be a limiting factor for the effectiveness of s.c. immunization rather than the generation of an effective T cell response. However, although we did not observe inhibition of the antitumor activity of the transferred T cells in the liver, the experimental data cannot exclude the possibility that the liver may constitute a suppressive environment for local activation of CTLs or for in vivo activated T cells.

Lymphocytes induced by s.c. immunization possess predominantly skin-homing capability

We next investigated whether s.c. immunization might induce T cells that preferentially migrate to s.c. tumor sites. We first examined whether the spleen cells obtained from s.c. immunized animals were enriched in effector or memory T cells displaying skin-homing receptors, CLA (27, 28), or CCR4 (29, 30), which would target them to cutaneous tissues. The spleen cells from the animals immunized s.c. with the GM/GP7TB vaccine were in vitro activated with irradiated GP7TB cells and then were analyzed for various surface markers. Table I reveals that most of the cells after stimulation were activated $CD3^+$ T lymphocytes and that they exhibited type 1 effector/memory cell characteristics, as they were

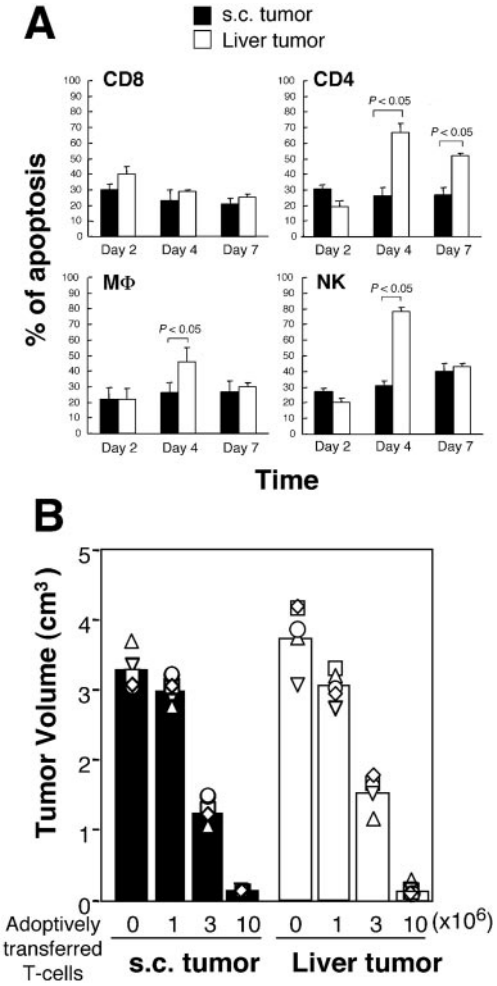

FIGURE 3. Enhanced apoptosis of some effectors observed at the liver tumor sites. *A*, Enhanced apoptosis. Mononuclear cells isolated from s.c. or liver tumors were stained with annexin V and an Ab against CD4, CD8, macrophages, or NK cells. The bars indicate mean cell numbers ± SD of five rats. The *p* values shown indicate where significantly different levels of apoptosis were observed between liver and s.c. tumor sites (Wilcoxon test). *B*, Equivalent antitumor activity of the adoptively transferred T cells at the s.c. and the liver tumor sites. Tumor-specific T cells isolated from the spleens of the s.c. GM/GP7TB-vaccinated animals were in vitro activated and then intratumorally injected into 3-day-old s.c. or liver tumors with the indicated cell numbers. Tumor sizes were measured on day 28 after tumor implantation.

Table I. *Characterization of the spleen cells stimulated with irradiated GP7TB cells and used for adoptive transfer*

Surface Markers	Spleen Cells from s.c. Immunization	Spleen Cells from Liver Immunization
CD3/CD25	95.9%	81.3%
CD4/CD25	56.59%	54.14%
CD8/CD25	33.56%	29.36%
IFN-γ	96.7%[a]/96.8%[b]	91.6%[a]/93.5%[b]
IL-4	4.5%[a]/6.5%[b]	9.9%[a]/9.3%[b]
$CD44/CD62L^{low}$	70.36%	63.2%

[a] Percent of total $CD4^+$ cells.
[b] Percent of total $CD8^+$ cells.

CD44/CD62L^{low} and secreted IFN-γ upon activation. Fig. 4 illustrates that the spleen cells from animals immunized s.c. with GM/GP7TB cells displayed significantly higher levels of CLA or CCR4 than did those from the control vaccine S2/GP7TB-treated animals. Most of these CLA$^+$ or CCR4$^+$ cells displayed activated T lymphocyte phenotypes as shown by expression of CD25. To further verify the skin-homing capability of these lymphocytes, the in vitro activated spleen cells were labeled with CFSE, and then injected via the tail vein to new experimental animals that harbored either an s.c. or an orthotopic liver tumor. Twenty-four hours later, CFSE$^+$ cells present in the s.c. tumors or the liver tumors were quantified by flow cytometry. An irrelevant tumor, RT-2, similarly inoculated in the liver or in the subcutis, was used as a control. Fig. 5*A* reveals that significant numbers of tumor-specific lymphocytes migrated to the s.c. GP7TB tumor sites, but much fewer cells migrated to the GP7TB tumors in the liver. Most of the CFSE$^+$ cells were T-lymphocytes (Fig. 5*B*). Similar s.c. homing capability was also observed for the splenocytes directly isolated after immunization without further in vitro activation, albeit at lower levels (data not shown). The numbers of T cells migrating to RT-2 tumors at the s.c. or the liver sites were low (Fig. 5*A*). These results thus confirm the preferential skin-homing capability of the effectors induced by s.c. immunization with the tumor cell vaccine.

Intrahepatic immunization with Ad-GM-CSF exhibited better therapeutic effects on orthotopic liver tumors than on s.c. or spleen tumors

We were interested to test the hypothesis that reversed antitumor effects might be observed if we changed the immunization site to the liver. Initial attempts to immunize the animals with irradiated tumor cell vaccines (3×10^7 cells) directly in the liver failed, however, because injection of so many cells in the liver caused rapid animal mortality. Therefore, we changed the immunization strategy. On day 0, animals were inoculated with tumors in one liver lobe, in the subcutis, or in the spleen. Three days later, 3×10^6 live tumor cells admixed with PBS, or 3×10^9 PFU Ad-GM-CSF or Ad-GFP, was used as a vaccine to immunize the animals in the other liver lobe. Tumor sizes were measured on day 28. Fig. 6 demonstrates that liver immunization of GP7TB cells mixed with Ad-GM-CSF significantly controlled the growth of orthotopic liver tumors compared with treatment with GP7TB cells mixed with PBS or Ad-GFP, but did not inhibit the growth of s.c. tumors or spleen tumors. In the control groups, liver immunization with

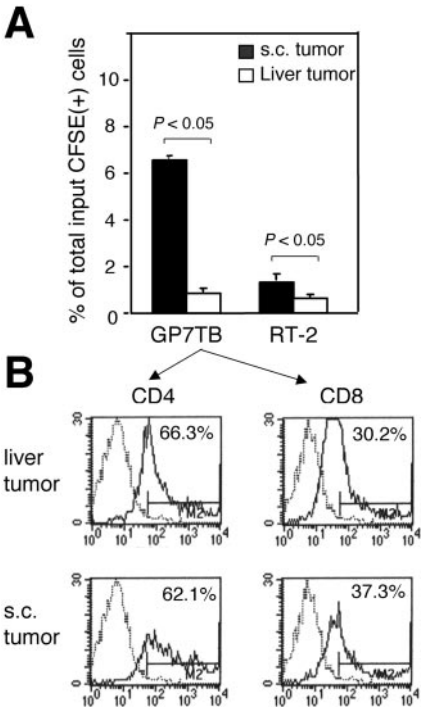

FIGURE 5. Preferential skin-homing capability of the effectors induced by s.c. immunization. *A*, Skin-homing. Effector cells isolated from the spleens of s.c. vaccine-treated animals were in vitro activated, CFSE labeled, and then injected via the tail vein to new animals bearing either s.c. or orthotopic liver tumors or irrelevant RT-2 tumors. CFSE$^+$ cells at both tumor sites were analyzed by flow cytometry 24 h after cell injection. The *p* values were obtained by the Wilcoxon test. *B*, The CFSE$^+$ cells isolated from the GP7TB tumors were further analyzed for expression of CD4 or CD8. Dotted lines indicate the cells stained with isotype-matched control Ab; solid lines show the cells stained with specific Ab as indicated. Most of the cells displayed T lymphocyte markers.

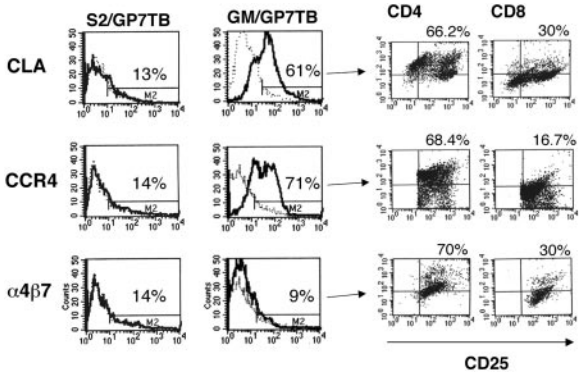

FIGURE 4. Elevated levels of skin-homing markers on the spleen cells obtained from the animals s.c. treated with the GM/GP7TB vaccine. The splenocytes of the animals treated s.c. with the GM/GP7TB or the S2/GP7TB vaccine were in vitro activated and then stained for the cutaneous-homing markers, CCR4 or CLA, or the gut-homing marker, $\alpha_4\beta_7$, plus the activation marker, CD25, and CD4 or CD8 marker. Dotted lines indicate the cells stained with isotype-matched control Ab; solid lines show the cells stained with specific Ab as indicated. The positive cells were further analyzed for activation and CD4 or CD8 markers, shown in the *right panels*.

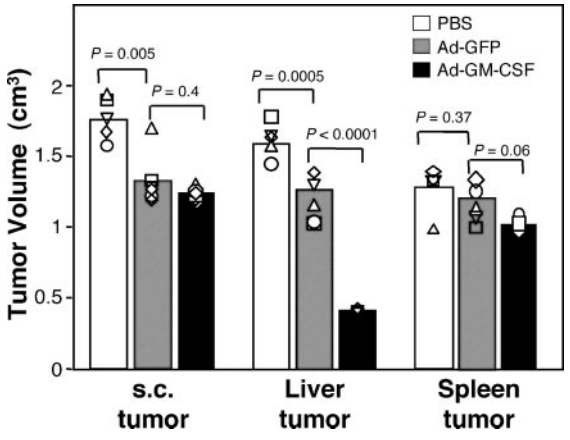

FIGURE 6. Better efficacy of intrahepatic immunization on orthotopic liver tumors compared with s.c. or spleen tumors. Rats bearing tumors in the subcutis, in one liver lobe, or in the spleen were treated 3 days later with 3×10^6 live GP7TB cells admixed with PBS, 3×10^9 PFU Ad-GFP, or 3×10^9 PFU Ad-GM-CSF in the other liver lobe. Tumor volumes were measured on day 28. Each group comprised five rats. The bars represent the mean tumor volume of each group. Statistical differences are indicated by *p* values (one-way ANOVA).

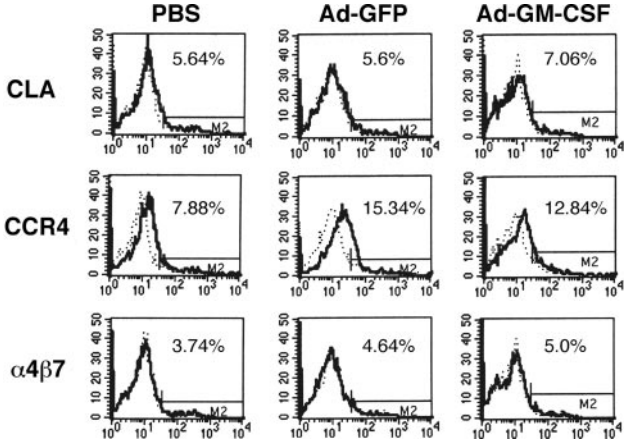

FIGURE 7. Low-level expression of skin- and gut-homing markers on the spleen cells of the animals intrahepatically immunized with tumor cell vaccines. The splenocytes isolated from the liver-immunized animals 25 days after immunization were in vitro stimulated and then stained for CCR4, CLA, or $\alpha_4\beta_7$.

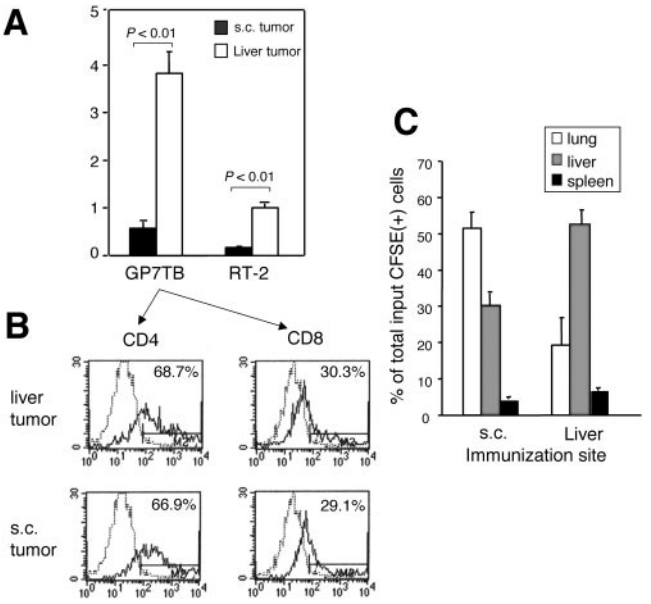

FIGURE 8. Preferential liver-homing capability of the effectors induced by intrahepatic immunization. Effectors were isolated from the spleens of the animals intrahepatically immunized with GP7TB mixed with Ad-GM-CSF, and adoptive transfer of CFSE-labeled cells was performed as described in Fig. 5. Distributions of $CFSE^+$ cells at both tumor sites (*A*) or in the lung, liver, or spleen organs (*C*) were determined by flow cytometry. The $CFSE^+$ cells in the s.c. and the liver GP7TB tumor sites were further analyzed for their T cell phenotypes (*B*).

GP7TB cells mixed with Ad-GFP also caused partial s.c. or liver tumor regression compared with treatment with GP7TB cells mixed with PBS, which probably was due to the bystander immunity induced by adenovirus infection.

Lymphocytes induced by liver immunization possess predominantly liver-homing capability

The spleen cells from the liver-immunized animals were examined for homing receptor expression. The lymphocytes from the animals immunized with GP7TB cells and Ad-GM-CSF displayed CLA or CCR4 at similar levels as found on lymphocytes isolated from the PBS or the Ad-GFP-immunized animals. Interestingly, they did not display high levels of the gut-homing marker, $\alpha_4\beta_7$, either (Fig. 7). Even so, these activated T cells, when adoptively transferred to new experimental animals, predominantly migrated to GP7TB tumors in the liver as opposed to GP7TB tumors in the subcutis (Fig. 8*A*). The $CFSE^+$ cells infiltrating into the tumor regions were mainly T lymphocytes (Fig. 8*B*). Interestingly, the number of $CFSE^+$ cells in the liver RT-2 tumors was also significantly greater than those in the s.c. RT-2 tumors (Fig. 8*A*). An opposite trend of distribution between the s.c. RT-2 tumors and the liver RT-2 tumors was observed when the transferred lymphocytes were isolated after s.c. immunization (Fig. 5). The results thus suggest that the T lymphocytes induced by different immunization routes have strong homing selection that also applies to nonspecific tumor sites. The organ distribution of CFSE-labeled lymphocytes derived from s.c. or liver immunized animals was also examined. Fig. 8*C* illustrates that, upon i.v. injection, the lymphocytes induced by s.c. immunization mainly migrated to the lungs, as seen in most situations in which cells are i.v. delivered, whereas a greater proportion of the lymphocytes induced by liver immunization migrated to the liver. Again, the majority of these $CFSE^+$ cells were T cells (data not shown). Collectively, these results suggest that liver immunization might activate a subset of T cells with unique liver homing capability, the markers of which, however, remain to be determined.

Discussion

The uniqueness of our study is the use of an orthotopic liver tumor model to investigate liver cancer immunotherapy. It has been shown that the local environment in different organs may affect the properties of tumors growing at those sites (31). In addition, the activity of some T cells or accessory cells may be altered by different microenvironments (32). Thus, an orthotopic tumor model is a clinically more relevant setting than an ectopic s.c. tumor model. In this study, we demonstrated that the growth of orthotopic liver tumors could be significantly controlled by intrahepatic immunization with a vaccine composed of tumor cells and Ad-GM-CSF, but not by s.c. immunization with an irradiated GM-CSF-secreting tumor cell vaccine. The s.c. immunization strategy elicited antitumor immunity that was effective against s.c. implanted tumors, but not against orthotopic liver or spleen tumors. The data further demonstrated that the T cells induced by s.c. immunization possessed preferential skin-homing capability, whereas those induced by liver immunization displayed preferential liver-homing capability. Taken together, our study clearly demonstrates that the site of immunization is critical for the control of local tumor growth.

Several studies using Ag-pulsed DCs as vaccines have reported that the route of immunization could impact antitumor efficacy (33–35). However, they did not report the existence of regionally distinct immune responses, nor did they systematically examine the control of tumors growing at different sites in the body. While this manuscript was in preparation, Mullins et al. (36) reported that i.v. immunization with DCs failed to impart protective immunity against s.c. tumors, but could control metastatic lung tumors. Yet, in their case, s.c. immunization imparted protection against both s.c. tumors and lung metastases. Collectively, their data combined with our study suggest that the immunization site and, thus, the regional draining LN where the initial APC-T cell interactions occur, plays a pivotal role in determining the distribution of the subsequently activated T cells.

Effector and memory T cells generally display selective tropism based on their ability to traffic through peripheral tissues such as inflamed skin or the intestinal lamina propria (17, 37). Tissue specificity is imparted by the use of different combinations of adhesion and chemokine molecules, which act together to capture, bind, and

then direct the migration of the lymphocytes into the tissues (17–19). The distinct combinations of endothelial adhesion molecules and chemokines in different tissues have been likened to a postal code, to which only lymphocytes expressing the appropriate receptors respond (38). For example, memory and effector cells recruited to inflamed skin sites predominantly express ligands for vascular P- or E-selectin (39). Particularly, CLA, a sialyl Lewis X-like carbohydrate epitope displayed on the P-selectin glycoprotein ligand-1 molecule, is expressed on almost 100% of skin-homing lymphocytes, allowing these T cells to roll on superficial dermal endothelium that express E-selectin (CD62E) (27, 28). CCR4, the receptor for the chemokine thymus and activation-regulation chemokine, is also found at high levels on skin-infiltrating lymphocytes, but not on intestine-infiltrating lymphocytes (29, 30), and is implicated in lymphocyte recruitment to normal and inflamed cutaneous sites. In contrast, mucosal lymphocytes are characterized by expression of integrin $\alpha_4\beta_7$, the receptor for the endothelial ligand, mucosal addressin cell adhesion molecule-1, which is largely restricted to gut vessels. The tissue-specific homing phenotypes of T cells are acquired in the regional draining LN soon (within 2 days) after immunization, as recently reported by Campbell and Butcher (40). In our study, the spleen lymphocytes isolated from animals treated with s.c. tumor cell vaccines displayed elevated levels of CLA and CCR4 (Fig. 4), strongly implicating their skin homing potentials. Upon adoptive transfer, these T cells mainly trafficked to the s.c. GP7TB tumor sites. This phenomenon explains why s.c. immunization was ineffective against orthotopic liver tumors.

More uniquely, the effector cells isolated from the liver-immunized animals did not express significant levels of CLA or CCR-4, nor did they express $\alpha_4\beta_7$. These T cells, however, displayed strong liver tumor and liver homing capability (Fig. 8, *A* and *C*). Previous reports have demonstrated the lack of mucosal addressin cell adhesion molecule-1 expression on hepatic endothelium and low numbers of lymphocytes positive for $\alpha_4\beta_7$ integrin in the liver, suggesting that in contrast with the gut, $\alpha_4\beta_7$ integrin does not play an important role in liver homing (41, 42). Furthermore, the sinusoids of the liver comprise an unique vesicular bed, which has a low-velocity blood flow and which, unlike other vascular beds, does not require capture mediated by selectins. Instead, a novel endothelial adhesion molecule, vascular adhesion protein-1, generally mediates the capture of lymphocytes in the liver (43). The receptor for vascular adhesion protein-1 is currently unknown. Our results are consistent with the notion that T lymphocyte recruitment to the liver might be different from the skin- or the gut-homing recirculation routes. It is unknown whether liver immunization allows primary activation of tumor-specific T cells by the many resident Kupffer cells present in the liver. In contrast, DCs in the liver may uptake tumor Ags, migrate to regional LN, and activate T cells that subsequently migrate back to the liver (44). Unfortunately, the factors that determine the infiltration of hepatic lymphocytes are still poorly understood. The unique observations made here suggest that further investigation of the liver-homing characteristics of T lymphocytes are warranted.

Although our data demonstrate that effector cells induced by s.c. immunization preferentially home to cutaneous tissues, some effector cells likely still migrate to the liver tumor region, albeit at low levels. As described by Mullins et al. (36), s.c. immunization with DCs protected animals from lung metastasis. In fact, our previous results showed that one dose of a live tumor cell vaccine (3×10^6 cells) administered s.c. effectively controlled the growth of 1-day-old orthotopic liver tumors. However, for 7-day-old tumors, s.c. immunization with a live vaccine was much less effective against orthotopic liver tumors compared with s.c. tumors (26). Therefore, we hypothesize that the homing characteristics of T lymphocytes induced by s.c. immunization are quantitative rather than qualitative. The "live" vaccines, owing to their proliferative capability, elicit much earlier and stronger antitumor immunity in the draining LN than do irradiated vaccines (26). Thus, it is postulated that sufficient numbers of activated T cells enter into the liver tumors to cause regression of the 1-day-old tumors, but not the 7-day-old tumors. By contrast, although the same proportion of activated T cells may migrate to the liver tumor sites after s.c. immunization of the "irradiated" tumor vaccine, the absolute number of activated T cells is insufficient to control liver tumor outgrowth. Similarly, the antitumor immunity induced by s.c. DC immunization as described by Mullins et al. (36) may also be strong enough to cause partial regression of lung metastases. The weak antitumor activity induced by the irradiated tumor cell vaccine in our study led to the recognition of the existence of regionally distinct immune responses induced by different immunization routes.

One unexpected observation noted in our study was the ineffectiveness of s.c. immunization or intrahepatic immunization on spleen tumor growth, despite significant levels of tumor-specific CTL activities that were detected in the spleens of the vaccinated animals (26). In fact, we previously found that the splenic CTL activity was usually undetectable until approximately day 14–35 after vaccination (data not shown). This is probably because the spleen is a secondary lymphoid organ, and the secondary immune responses occur much later than the primary responses in the draining LN (26). Early infiltration of CTLs into the tumor region is critical for efficient regression of tumors when they are still small (26). Compared with the liver tumors treated by s.c. immunization (Fig. 1) or the s.c. tumors treated by intrahepatic immunization (Fig. 6), the spleen tumors responded somewhat better to either immunization strategy. So we believe that tumor-specific T cells initially activated in the draining LN may not efficiently migrate to the spleen at early times. The CTLs present at later times in the spleen may be ineffective due to the large sizes of the tumors.

In summary, our study provides strong evidence demonstrating that different sites of tumor cell vaccination can induce distinct populations of T cells that display different homing phenotypes and are differentially involved in controlling the outgrowth of tumors at different sites in the body. The results have significant clinical implications, suggesting that the route of immunization should be considered as an important variable when designing immunotherapy protocols for regional orthotopic tumors. They also provide a basis for future understanding of T cell homing to the liver.

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