Reduced Efficacy of Allogeneic versus Syngeneic Fibroblasts Modified to Secret Cytokines as a Tumor Vaccine Adjuvant

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Division of Surgical Oncology, University of Michigan, Ann Arbor, Michigan 48109

ABSTRACT

We examined the relative efficacy of allogeneic versus syngeneic fibroblasts admixed with tumor cells as a vaccine to induce antitumor T-cell reactivity. Allogeneic (3T3) or syngeneic (BLK) fibroblasts transfected to secrete equivalent amounts of GM-CSF were admixed with either D5 melanoma or MCA 207 sarcoma and inoculated s.c. into the flanks of C57BL/6 mice. Vaccine-primed lymph node (LN) cells were examined for in vivo antitumor reactivity in an adoptive transfer model. At fibroblast: tumor cell ratios of ≤1, allogeneic and syngeneic granulocyte macrophage colony-stimulating factor-secreting fibroblasts enhanced T-cell reactivity to tumor cells. However, at ratios of 2.4, the adjuvant effect induced by granulocyte macrophage colony-stimulating factor-stimulating fibroblast was not evident. Instead, we observed increased alloreactivity of primed LN cells against 3T3 targets as assessed by cytotoxicity and cytokine release assays, which was not observed with syngeneic fibroblasts. Moreover, with increasing numbers of allogeneic fibroblasts, there was a skewing of the T-cell Vß repertoire. These latter cells responded to tumor stimulation with the release of greater amounts of interleukin 10, which may account for the diminished antitumor reactivity observed in vivo. Allogeneic fibroblasts transfected to secrete interleukin 2 or IFN-γ also induced diminished tumor reactivity of primed LN cells. Syngeneic fibroblasts are superior to allogeneic fibroblasts as vehicles to deliver cytokines in tumor vaccines.

INTRODUCTION

The local delivery of various cytokines at the site of a tumor inoculum has been reported to result in altered tumorigenicity and up-regulated host immunity to tumor antigens (1, 2). The majority of these approaches have used gene-modified tumor cells that act in an autocrine fashion to release cytokine in the tumor microenvironment. The enhanced immunogenicity displayed by these cytokine-secreting tumor cells has resulted in the successful treatment of established tumors in selected models when they have been used as a vaccine (3–5). The use of autologous human tumor cells transduced to secrete different cytokines is currently being investigated in clinical trials (6).

A significant impediment to the clinical application of genetically modified autologous tumor cells as a vaccine is the reliability of generating such cells. Depending on the method of transfection, (i.e., retrovirus) a cultured tumor line may be required. This may not be always feasible. An alternative is the use of cytokine-secreting fibroblasts that can be admixed with tumor cells. In animal models, this approach has been successful in developing enhanced host immunity to tumor cells (7, 8). These studies have demonstrated the important principle that the paracrine delivery of cytokines can elicit a similar immune response compared with gene-modified tumor cells. Fibroblasts are available in a clinical setting from a skin biopsy and can easily be cultured. Clinical studies using cytokine-secreting fibroblasts are also being conducted in cancer patients (9, 10).

In this report, we have evaluated the relative efficacy of using allogeneic versus syngeneic fibroblasts transfected to secrete cytokines as an adjuvant to tumor vaccination. We have reported previously in animal models that tumor cells transfected to secrete GM-CSF will enhance reactivity to wild-type tumor cells of T cells derived from TDLNs (11, 12). This phenomenon is apparent in both weakly immunogenic (13) and poorly immunogenic murine tumor systems (11, 13). We found that the use of either allogeneic or syngeneic fibroblasts transfected to secrete GM-CSF will enhance T-cell tumor reactivity. However, at increasing ratios of fibroblast: tumor cells in the vaccine, allogeneic fibroblasts resulted in diminished adjuvant activity. Our studies further characterized the potential mechanisms associated with this phenomenon. The use of allogeneic cytokine-secreting fibroblasts is less effective than that of syngeneic fibroblasts in the application of tumor vaccines for active, specific immunotherapy.

MATERIALS AND METHODS

Mice. Female B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free environment. They were used at the age of 8 weeks or older. Recognized principles of laboratory animal care were followed, and all animal protocols were approved by the University of Michigan Laboratory of Animal Medicine.

Tumors. The B16 melanoma is a tumor of spontaneous origin that has been studied extensively (15). A subclone of the B16-BL6 tumor, termed D5, has been characterized previously by our laboratory (11). Tumor cells were maintained by serial in vitro passage in CM, which consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS, 0.01 mm nonessential amino acids, 1 mm sodium pyruvate, 2 mm L-glutamine, 100 ìg/ml streptomycin, 100 IU/ml penicillin, 50 ìg/ml gentamicin, and 10−6 m 2-mercaptoethanol. For i.v. and s.c. tumor inoculation, adherent tumor cells were removed from the flask using trypsin and washed in CM. Tumor cells were washed in HBSS for administration into animals.

MCA 207 is a fibrosarcoma syngeneic to B6 mice induced by 3-methylcholanthrene and were provided kindly by Dr. James C. Yang (National Cancer Institute, NIH, Bethesda, MD; Ref. 16). Tumor cell suspensions were maintained in vivo by serial s.c. transplantation in syngeneic mice and were used within the seventh transplantation generation. Tumor cell suspensions were prepared from solid tumors by enzymatic digestion in 50 ml of HBSS (Life Technologies, Inc.) containing 40 mg of collagenase, 4 mg of DNase, and 100 IU of hyaluronidase (Sigma Chemical Co., St. Louis, MO) for 3 h at room temperature as described previously (17).

Genetic Modification of Fibroblasts. Both allogeneic 3T3 and syngeneic BLK fibroblast lines were obtained from American Type Culture Collection (Rockville, MD). 3T3 cells were derived originally from the NIH-Swiss mouse strain that is not of an H-2β background. BLK fibroblasts were derived from the B6 host, which is composed of the H-2β background. Cytokine-secreting fibroblasts were generated using retroviral vectors containing a MFG backbone. Vectors encoding murine GM-CSF, murine IFN-γ, and murine IL-2 were constructed as described previously (13). Cytokine-secreting fibroblasts were generated by splitting confluent cells 1:10 and transducing them with
Cytokine-Assessing Fibroblasts as Vaccine Adjuvants

TDLN cells were cultured in 2 ml of CM at 37°C in a 5% CO2 incubator for foci too numerous to count were assigned an arbitrary value of >250. The a 15% solution of India ink and bleached by Fekete's solution (20). D5 nodules appeared as discrete, white nodules on the black surface of lungs insufflated with D5 or MCA 207 tumor cells to establish pulmonary metastases. Three days after tumor inoculation, mice were injected iv. with TDLN cells. At approx. 3 weeks after tumor initiation, mice were randomized and sacrificed. The GM-CSF-secreting BLK, BLK-GM, secreted 50 ng/ml/24 h of the murine GM-CSF, a standard curve starting at 20 ng/ml with 11 serial 2-fold dilutions was performed. For IFN-γ and IL-10, standard curves starting at 1000 IU/ml and 20 ng/ml, respectively, were established in a similar fashion in 24-well tissue culture plates. After 24 h, culture supernatants were collected for cytokine measurements performed in duplicate using commercially available ELISA. If detectable, background cytokine values produced by tumors alone were subtracted from the coculture values when reporting results.

RESULTS

In Vivo Efficacy of GM-CSF-secreting Fibroblasts as an Adjuvant to D5 Melanoma Tumor Priming. In our initial studies, we observed that both allogeneic and syngeneic fibroblasts modified to secrete GM-CSF will induce increased LN cell numbers. Animals were inoculated s.c. with D5 tumor cells admixed with increasing numbers of GM-CSF-secreting fibroblasts, and draining LN cells were harvested 9 days later. As summarized in Table 1, mean cell yield per LN was not altered with the admixture of allogeneic (i.e., D3T) or syngeneic (i.e., BLK) fibroblasts that did not secrete GM-CSF. However, the mean cell yield per LN rose with increasing numbers of admixed GM-CSF-secreting fibroblasts (i.e., D3T-GM or BLK-GM). There was a 3-fold increase in cell yield with the highest number of cytokine-secreting fibroblasts admixed with tumor cells at a ratio of 2.4:1, respectively. There were no differences in cell yield between equivalent numbers of GM-CSF-secreting allogeneic or syngeneic fibroblasts. Flow cytometry of the freshly harvested LNs draining inocula of tumor cells admixed with 3T3-GM fibroblasts was performed for T cells (i.e., CD3, CD4, and CD8) and macrophage/neutrophil (i.e., Mac-1 and Mac-2) markers. The percentage of Mac-1- and Mac-2-positive cells was unchanged at <1% for all of the LN groups. However, we did observe an increasing CD4:CD8 ratio in the draining LNs, when greater numbers of 3T3-GM fibroblasts were admixed with tumor cells. LNs draining tumor cells alone, or admixed with 0.6 × 10^6, 1.0 × 10^6, or 2.4 × 10^6 3T3-GM fibroblasts, exhibited CD4:CD8 ratios of 2.3, 3.2, 3.4, and 4.4, respectively. This represents an absolute increase in the CD4+ subset of T cells within the draining LNs induced by 3T3-GM cells. Flow analysis of LN cells

<p>| Table 1 Lymph node cell number increases after vaccination with tumor admixed with fibroblasts secreting GM-CSF |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Fibroblasts</th>
<th>D5 tumor</th>
<th>Mean cell number per LN (× 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>3T3-GM</td>
<td>1.0</td>
<td>+</td>
</tr>
<tr>
<td>3T3-GM</td>
<td>0.6</td>
<td>+</td>
</tr>
<tr>
<td>3T3-GM</td>
<td>1.0</td>
<td>+</td>
</tr>
<tr>
<td>BLK-GM</td>
<td>2.4</td>
<td>+</td>
</tr>
<tr>
<td>BLK-GM</td>
<td>0.8</td>
<td>+</td>
</tr>
<tr>
<td>BLK-GM</td>
<td>2.4</td>
<td>+</td>
</tr>
</tbody>
</table>

B6 mice inoculated with 10^6 D5 tumor cells admixed with fibroblasts s.c., with five mice per group. a Draining LNs were harvested 9 days after vaccination, and cells were counted. b+, groups of mice inoculated with tumor.

Cytokine-Assessing Fibroblasts as Vaccine Adjuvants

mL of virus in the presence of Polybrene (8 μg/ml). Transduced tumor cell clones were established by the limiting dilution technique. Cytokines were measured by ELISAs, and the highest cytokine-secreting clones were used for study. The GM-CSF-secreting BLK, BLK-GM, secreted 50 ng/ml/24 h of the transgenic protein. A comparable GM-CSF-secreting 3T3 line, 3T3-GM, was isolated. 3T3 lines transduced to secrete IFN-γ (3T3-IFN-γ) and IL-2 (3T3-IL-2) produced 150 and 120 IU/ml/24 h of the transgenic proteins, respectively.

Cytokine Assays. Commercially available ELISA methods were used to measure GM-CSF, IFN-γ, and IL-2 (PhanMingen, San Diego, CA). For murine GM-CSF, a standard curve starting at 20 ng/ml with 11 serial 2-fold dilutions was performed. For IFN-γ and IL-10, standard curves starting at 1000 IU/ml and 20 ng/ml, respectively, were established in a similar fashion in 24-well tissue culture plates. After 24 h, culture supernatants were collected for cytokine measurements performed in duplicate using commercially available ELISA. If detectable, background cytokine values produced by tumors alone were subtracted from the coculture values when reporting results.

In Vitro Cytokine Release. After in vitro activation, TDLN cells were restimulated with irradiated autologous tumor cells in CM. Tumor stimulator cells were irradiated by a 137Cs source before using (7000 Gy for MCA 207 and 30,000 Gy for D5). LN cells (10^6/ml) and irradiated stimulators (5 × 10^6/ml) were cocultured in 2-ml flasks in 24-well tissue culture plates. After 24 h, culture supernatants were collected for cytokine measurements performed in duplicate using commercially available ELISA. If detectable, background cytokine values produced by tumors alone were subtracted from the coculture values when reporting results.

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<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>3T3-GM</td>
<td>1.0</td>
<td>+</td>
</tr>
<tr>
<td>3T3-GM</td>
<td>0.6</td>
<td>+</td>
</tr>
<tr>
<td>3T3-GM</td>
<td>1.0</td>
<td>+</td>
</tr>
<tr>
<td>BLK-GM</td>
<td>2.4</td>
<td>+</td>
</tr>
<tr>
<td>BLK-GM</td>
<td>0.8</td>
<td>+</td>
</tr>
</tbody>
</table>

B6 mice inoculated with 10^6 D5 tumor cells admixed with fibroblasts s.c., with five mice per group. a Draining LNs were harvested 9 days after vaccination, and cells were counted. b+, groups of mice inoculated with tumor.
TDLN cells were transferred to secondary hosts with established 3-day lung metastases. Unlike with 313-GM, there was no evidence of inhibition of LN cell priming by the highest fibroblast:tumor ratio. The combination of non-cytokine-secreting syngeneic and allogeneic cytokine-secreting fibroblasts was not performed.

In a series of four experiments, the therapeutic efficacy of TDLN cells primed in vivo to D5 tumor cells admixed with GM-CSF-secreting fibroblasts was assessed. D5 tumor cells admixed with equivalent numbers of syngeneic or allogeneic cytokine-secreting fibroblasts were inoculated s.c., and TDLN cells were harvested 9 days later for anti-CD3/IL-2 activation. The in vitro antitumor reactivity of the cells was assessed by adoptive transfer into mice with established pulmonary metastases. In experiments 1 and 2 (Table 2), D5 tumor cells alone induced a minimal antitumor response in the draining LNs, as indicated by a modest reduction in the mean number of lung metastases. The combination of non-cytokine-secreting BLK syngeneic fibroblasts and D5 tumor cells did not influence the priming of TDLN cells. However, the admixture of GM-CSF-secreting BLK fibroblasts and D5 tumor cells did result in maximal tumor reactivity in both experiments. In experiment 3 (Table 2), non-cytokine-secreting allogeneic or syngeneic 3T3 fibroblasts did not influence the modest priming of TDLN cells by D5 tumor cells alone. However, the combination of D5 and GM-CSF-secreting 3T3 fibroblasts resulted in enhanced tumor-reactivity in both experiments. In experiment 4, the relative efficacies of cytokine-secreting syngeneic and allogeneic fibroblasts were examined in the same experiment. We found that syngeneic BLK fibroblasts were significantly better than allogeneic 3T3 fibroblasts in the induction of tumor-primed LN cells.

To assess the relative effect of allogeneic fibroblasts in tumor priming, we increased the number of GM-CSF-secreting fibroblasts (3T3-GM) admixed with D5 tumor cells admixed with a vaccine. The vaccine was inoculated s.c., and TDLN cells were harvested and assessed for in vivo antitumor reactivity, as described previously. As shown in Table 3, when ratios of 3T3-GM:D5 tumor cells were given at 0.15 and 0.6, there was significant induction of immune TDLN cells. However, at a ratio of 2.4, there was inhibited tumor reactivity in the draining LNs.

A similar assessment of syngeneic fibroblasts was performed. GM-CSF-secreting B6 fibroblasts (BLK-GM) were admixed with D5 tumor cells at increasing ratios, and the sensitization of draining LN cells was evaluated in the adoptive immunotherapy model. As summarized in Table 4, the admixture of BLK-GM with D5 cells in ratios of 0.8 and 2.4 resulted in significant priming of TDLN cells compared to no fibroblasts. Unlike with 3T3-GM, there was no evidence of inhibition of LN cell priming at the highest fibroblast:tumor ratio.

In Vitro Immune Function of LN Cells Primed with D5 Tumor Cells Admixed with GM-CSF-secreting Fibroblasts. We proceeded to assess the in vitro immunological function of vaccine-primed TDLN cells derived by methods described in the previous section. Animals were primed in vivo by the s.c. inoculation of D5 tumor cells admixed with 3T3, BLK, 3T3-GM, or BLK-GM fibroblasts in equal ratios with TDLN cells harvested 9 days later for anti-CD3/IL-2 activation. A 48-h cytotoxicity assay was used to assess the reactivity of activated TDLN cells against three different targets: D5 tumor cells, 3T3 fibroblasts, and BLK fibroblasts. As demonstrated in Fig. 1, TDLN cells primed by D5 tumor cells admixed with GM-CSF-secreting fibroblasts mediated increased cytotoxicity against D5 tumor targets compared with the other groups of effector cells. There was no difference in cytotoxicity profiles against D5 tumor cells among TDLN cells primed with D5 tumor cells alone or in combination with non-cytokine-secreting allogeneic or syngeneic fibroblasts. These observations demonstrated up-regulated antitumor response due to the local elaboration of GM-CSF. Cytotoxicity

Table 2 Therapeutic efficacy of TDLN cells primed in vivo with D5 tumor cells admixed with allogeneic versus syngeneic GM-CSF-secreting fibroblasts

<table>
<thead>
<tr>
<th>Group</th>
<th>Source of TDLNs</th>
<th>In vivo IL-2</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>-</td>
<td>&gt;250</td>
<td>203 (10)</td>
<td>&gt;250</td>
<td>203 (10)</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>+</td>
<td>179 (8)</td>
<td>232 (8)</td>
<td>179 (8)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>D5</td>
<td>+</td>
<td>156 (25)</td>
<td>97 (7)</td>
<td>152 (25)</td>
<td>97 (7)</td>
</tr>
<tr>
<td>D</td>
<td>D5 + BLK</td>
<td>+</td>
<td>148 (32)</td>
<td>109 (8)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>D5 + BLK-GM</td>
<td>+</td>
<td>0&quot;</td>
<td>0&quot;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>D5 + 3T3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>176 (32)</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>D5 + 3T3-GM</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>44 (9)</td>
<td>32 (10)</td>
</tr>
</tbody>
</table>

The in vitro antitumor reactivity of the cells was assessed by adoptive transfer into mice with established pulmonary metastases. In experiments 1 and 2 (Table 2), D5 tumor cells alone induced a minimal antitumor response in the draining LNs, as indicated by a modest reduction in the mean number of lung metastases. The combination of non-cytokine-secreting BLK syngeneic fibroblasts and D5 tumor cells did not influence the priming of TDLN cells. However, the admixture of GM-CSF-secreting BLK fibroblasts and D5 resulted in maximal tumor reactivity in both experiments. In experiment 3 (Table 2), non-cytokine-secreting allogeneic 3T3 fibroblasts did not influence the modest priming of TDLN cells by D5 tumor cells. However, the combination of D5 and GM-CSF-secreting 3T3 cells did result in enhanced TDLN sensitization. In experiment 4, the relative efficacies of cytokine-secreting syngeneic and allogeneic fibroblasts were examined in the same experiment. We found that syngeneic BLK fibroblasts were significantly better than allogeneic 3T3 fibroblasts in the induction of tumor-primed LN cells.

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Table 3 Diminished therapeutic efficacy of TDLN cells primed in vivo with D5 tumor cells admixed with increasing numbers of allogeneic GM-CSF-secreting fibroblasts

<table>
<thead>
<tr>
<th>Group</th>
<th>Source of TDLNs</th>
<th>Mean no. of lung metastases (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>226 (15)</td>
</tr>
<tr>
<td>B</td>
<td>+ 0</td>
<td>215 (21)</td>
</tr>
<tr>
<td>C</td>
<td>+ 1.5 x 10^6</td>
<td>60 (10)</td>
</tr>
<tr>
<td>D</td>
<td>+ 6.0 x 10^6</td>
<td>64 (14)</td>
</tr>
<tr>
<td>E</td>
<td>+ 2.4 x 10^6</td>
<td>230 (11)</td>
</tr>
</tbody>
</table>

B6 mice were inoculated with 10^6 D5 tumor cells admixed with various numbers of allogeneic 3T3-GM fibroblasts. TDLNs were harvested 9 days later, activated in vitro, and transferred as described in Table 3. —, not given; +, given.

Table 4 Therapeutic efficacy of TDLN cells primed in vivo with D5 tumor cells admixed with increasing numbers of syngeneic GM-CSF-secreting fibroblasts

<table>
<thead>
<tr>
<th>Group</th>
<th>Source of TDLNs</th>
<th>Mean no. of lung metastases (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>&gt;250</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>&gt;250</td>
</tr>
<tr>
<td>C</td>
<td>+ 0</td>
<td>118 (20)</td>
</tr>
<tr>
<td>D</td>
<td>+ 8 x 10^6</td>
<td>0&quot;</td>
</tr>
<tr>
<td>E</td>
<td>+ 2.4 x 10^6</td>
<td>0&quot;</td>
</tr>
</tbody>
</table>

B6 mice were inoculated with 10^6 D5 tumor cells admixed with various numbers of syngeneic BLK-GM fibroblasts. TDLNs were harvested 9 days later, activated in vitro, and transferred as described in Table 3. —, not given; +, given.

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against 3T3 targets was enhanced significantly by TDLN cells primed in vivo with D5 admixed with 3T3 or 3T3-GM (Fig. 1). This represented a specific immune response to alloantigens expressed by 3T3 cells, because these same TDLN cells did not manifest enhanced cytotoxicity against syngeneic BLK fibroblasts. As expected, the use of syngeneic BLK or BLK-GM fibroblasts admixed with D5 did not induce enhanced cytotoxicity of TDLN cells against allogeneic or syngeneic fibroblast targets. Due to the long-term culture of the cytotoxicity assay used, there was nonspecific lysis observed with the three targets used.

The release of cytokines by T cells in response to tumor stimulation has been documented to be associated with in vivo tumor reactivity in adoptive transfer (22–24). We next examined the cytokine profiles released by TDLN cells primed with D5 admixed with 3T3-GM or BLK-GM. Mice were inoculated with D5 tumor cells admixed with GM-CSF-secreting fibroblasts at fibroblast:tumor ratios of 0.8 (low) or 2.4 (high), and TDLN cells were harvested 9 days later for anti-CD3/IL-2 activation. After activation, the TDLN cells were stimulated in vitro with irradiated 3T3 or BLK fibroblasts, and the amount of cytokine was released after 24 h was measured. As summarized in Fig. 2, TDLN cells primed by D5 tumor cells alone released negligible amounts of GM-CSF, IFN-\(\gamma\), and IL-10 in response to either 3T3 or BLK fibroblast stimulators. However, there were significant amounts of cytokine released by TDLN cells primed with D5 admixed with 3T3-GM in response to 3T3 stimulator cells. The cytokine response was greater with increasing numbers of 3T3-GM cells used to prime mice and specific because it was not evident with BLK stimulator cells. By contrast, there was no release of cytokines by activated TDLN cells primed with D5 admixed with syngeneic BLK-GM fibroblasts in the same assay in which BLK stimulator cells were used. These results demonstrated significant induction of immune reactivity to 3T3 alloantigens. As will be shown in subsequent studies, this...
activation. Two X 10^6 cells were transferred iv. into secondary mice with established 3-day lung metastases. No IL-2 was administered.

BLK or BLK-GM fibroblasts. TDLNs were harvested 9 days later for anti-CD3/IL-2 vant to MCA 207 Sarcoma. We examined the relative efficacy of response has influenced the T-cell repertoire in response to tumor

Table 5 Therapeutic efficacy of TDLN cells primed in vivo with MCA 207 tumor cells admixed with syngeneic GM-CSF-secreting fibroblasts

<table>
<thead>
<tr>
<th>Group</th>
<th>MCA 207</th>
<th>No. of BLK fibroblasts</th>
<th>No. of BLK-GM fibroblasts</th>
<th>Mean no. of lung metastases (SE)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>186 (24)</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>8 X 10^3</td>
<td>−</td>
<td>56 (6)^a</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>8 X 10^3</td>
<td>−</td>
<td>62 (8)^c</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>8 X 10^3</td>
<td>+ 2.4 X 10^6</td>
<td>8 (2)^f</td>
</tr>
</tbody>
</table>

a B6 mice were inoculated s.c. with 10^6 MCA 207 tumor cells admixed with either BLK or BLK-GM fibroblasts. TDLNs were harvested 9 days later for anti-CD3/IL-2 activation. Two X 10^6 cells were transferred i.v. into secondary mice with established 3-day lung metastases. No IL-2 was administered.

b P < 0.0001 compared to group A; P < 0.01 compared to groups B and C.

c P < 0.0001 compared to group A; P < 0.01 compared to groups B and C.

d P < 0.0001 compared to group A.

e P < 0.0001 compared to group A; P < 0.05 compared to groups B and C.

response has influenced the T-cell repertoire in response to tumor cells as well.

In Vivo Efficacy of GM-CSF-secreting Fibroblasts as an Adjuvant to MCA 207 Sarcoma. We examined the relative efficacy of syngeneic and allogeneic GM-CSF-secreting fibroblasts as an adjuvant in priming TDLN cells to MCA 207. Unlike D5, the MCA 207 is an immunogenic tumor that can regress in vivo in response to high-dose IL-2 administration. In our initial studies, we examined whether syngeneic GM-CSF-secreting fibroblasts admixed with MCA 207 would enhance the induction of pre-effector TDLN cells. Using a similar model, as described previously with the D5 melanoma, TDLN cells were harvested after in vivo priming and activated by the anti-CD3/IL-2 method. The antitumor reactivity of the activated cells was then assessed by the adoptive transfer into mice bearing 3-day pulmonary metastases. As shown in Table 5, MCA 207 tumor cells induced immunocompetent TDLN cells capable of mediating tumor regression. The admixture of non-cytokine-secreting BLK fibroblasts with MCA 207 tumor cells did not influence the priming of TDLN cells. However, the admixture of BLK-GM resulted in increased induction of pre-effector TDLN cells that manifested a greater reduction of lung tumor. This effect appeared to be related to the amount of BLK-GM present because increased numbers of cells resulted in greater antitumor reactivity. These studies extend our observations with the D5 tumor cells and indicate that GM-CSF delivered by syngeneic fibroblasts has a significant adjuvant effect in a different tumor system.

In a similar fashion, 3T3-GM fibroblasts were examined for their adjuvant effect on the priming of LN cells with MCA 207. MCA 207 tumor cells were admixed with increasing amounts of 3T3-GM fibroblasts. The activated TDLN cells were evaluated for antitumor reactivity in the adoptive transfer model. As shown in Table 6, the combination of MCA 207 tumor cells and 3T3-GM fibroblasts at ratios of 0.15 and 0.6 resulted in enhanced priming of TDLN cells compared to tumor cells used alone. However, this adjuvant effect was lost when the ratio of fibroblast:tumor was increased to 2.4. As with the D5 tumor cells, larger numbers of allogeneic GM-CSF-secreting fibroblasts had an inhibitory effect in the induction of tumor immunity.

In Vitro Immune Function of LN Cells Primed with MCA 207 Tumor Cells Admixed with GM-CSF-secreting Fibroblasts. We analyzed the function of MCA 207–primed LN cells with the long-term cytotoxicity assay and cytokine release assays. As with the D5 tumor model, GM-CSF-secreting fibroblasts admixed with MCA 207 enhanced cytotoxicity of primed TDLN cells against MCA 207 targets compared to TDLN cells primed with MCA 207 alone (data not shown). For cytokine release analysis, MCA 207 tumor cells were admixed with increasing numbers of 3T3-GM fibroblasts at fibroblast: tumor cell ratios of 0.15 (low), 0.6 (medium), and 2.4 (high) for priming of LN cells. Activated TDLN cells were then assessed for release of IFN-γ, GM-CSF, and IL-10 in response to MCA 207 or 3T3 stimulator cells. As shown in Fig. 3, there were significant amounts of all three cytokines released by the different TDLN populations in response to MCA 207 stimulator cells. When 3T3 cells were used as a stimulator in the assay, the amount of all three cytokines released increased with greater numbers of 3T3-GM cells used to prime LNs. As seen previously with the D5 tumor model, MCA 207 tumor cells admixed with syngeneic BLK-GM fibroblasts did not elicit TDLN cells responsive to BLK stimulator cells in the cytokine release assays (Fig. 4). These experiments substantiate a significant allogeneic immune response associated with the coinoculation of 3T3 fibroblasts with MCA 207 tumor cells.

To further characterize the effect of 3T3-GM on tumor priming of LN cells, we enriched T-cell subsets for selected Vβ in the primed LN and analyzed cytokine release. In prior studies, we have documented that five subpopulations based on Vβ usage constitute a majority of MCA 207 TDLN cells after anti-CD3/IL-2 activation and include: Vβ3, Vβ5, Vβ7, Vβ8, and Vβ11 (25). Using specific anti-Vβ mAbs, we can activate these T-cell subsets in culture and selectively expand them in IL-2 to obtain enriched (>90%) Vβ+ cells for analysis (25). Using this technique, we compared the cytokine release of TDLN cells primed by MCA 207 alone versus tumor cells admixed with 3T3-GM at a high ratio of fibroblast:tumor (i.e., 2.4). The results are summarized in Fig. 5. The relative amounts of IFN-γ and GM-CSF released in response to the different Vβ subsets was diminished from TDLN cells primed with tumor combined with 3T3-GM compared with MCA 207 alone. We have documented previously that Vβ8+ cells are the predominant effector population mediating tumor regression in the MCA 207 model (25). In the current study, Vβ8+ cells primed in vivo with MCA 207 and 3T3-GM cells expressed decreased IFN-γ and GM-CSF in response to MCA 207 alone. These same cells responded to allogeneic 3T3 with increased IFN-γ and GM-CSF, indicating their ability to respond to alloantigens.

Of significant interest was the release profile of IL-10 expressed by the different Vβ subsets. There was an increase of IL-10 released in response to tumor stimulation by all five subsets primed by tumor cells admixed with 3T3-GM. For the Vβ3+, 5+, 7+, and 8+ subpopulations, there was a 4-fold increase of IL-10 release to MCA 207 stimulation (Fig. 5). All five subpopulations also released significant amounts of IL-10 in response to 3T3 stimulation, which was absent for TDLN cells primed with tumor cells alone. The percentage of these Vβ8 subpopulations was not significantly different between TDLN cells primed with tumor cells alone versus tumor cells admixed

Table 6 Diminished therapeutic efficacy of TDLN cells primed in vivo with MCA 207 tumor cells admixed with increasing numbers of allogeneic GM-CSF-secreting fibroblasts

<table>
<thead>
<tr>
<th>Group</th>
<th>MCA 207</th>
<th>No. of 3T3-GM fibroblasts</th>
<th>Mean no. of lung metastases (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>−</td>
<td>−</td>
<td>&gt;250</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>1.5 X 10^3</td>
<td>46 (9)^c</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>6.0 X 10^3</td>
<td>12 (6)^c</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>2.4 X 10^6</td>
<td>42 (11)^c</td>
</tr>
</tbody>
</table>

a B6 mice were inoculated s.c. with 10^6 MCA 207 tumor cells admixed with increasing numbers of 3T3-GM fibroblasts. TDLNs were harvested 9 days later for anti-CD3/IL-2 activation. Two X 10^6 activated TDLN cells were transferred i.v. into secondary hosts with established 3-day lung metastases. All mice received IL-2 (60,000 IU i.p.) twice daily, with eight doses after cell transfer.

b P < 0.001 compared to group A.

c P < 0.001 compared to group A; P < 0.05 compared to groups B and C.

d P < 0.0001 compared to group A; P < 0.01 compared to groups B and C.

e P < 0.05 compared to groups B and C.

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with allogeneic fibroblasts. The percentages of selected Vβ subpopulations of the whole population of TDLN cells primed in vivo with MCA 207 tumor cells alone were: Vβ3+, 3.1%; Vβ5+, 15.1%; Vβ7+, 7.6%; Vβ8+, 26.6%; and Vβ11+, 11.2%. Similarly, the Vβ subpopulations of the whole population of TDLN cells primed in vivo with MCA 207 tumor cells admixed with 3T3-GM fibroblasts consisted of: Vβ3+, 3.2%; Vβ5+, 15.7%; Vβ7+, 8.2%; Vβ8+, 24.6%; and Vβ11+, 9.3%. As will be discussed, we believe that an altered cytokine profile released by TDLN cells, rather than a skewing of the Vβ repertoire to a dominant subpopulation, is directly responsible for the changes in the in vivo tumor reactivity measured in our adoptive transfer experiments.
Allogeneic Fibroblasts Modified to Secrete IFN-γ or IL-2 as an Adjuvant to MCA 207 Tumor Priming. We have constructed other 3T3 fibroblast lines that secrete IL-2 and IFN-γ. These fibroblasts were examined in the MCA 207 model for their adjuvant effect in priming TDLN cells. 3T3 fibroblasts were admixed with MCA 207 tumor cells at a fibroblast:tumor ratio of 1. TDLN cells were evaluated subsequently for antitumor reactivity by adoptive transfer into mice with MCA 207 lung metastases. As summarized in Table 7, the admixture of wild-type allogeneic fibroblasts reduced the antitumor reactivity of TDLN cells primed by MCA 207. The paracrine secretion of GM-CSF resulted in enhanced tumor reactivity of the TDLN cells compared to MCA 207 alone or MCA 207 admixed with 3T3 fibroblasts, as anticipated from our previous experiments. However, the elaboration of IFN-γ or IL-2 by 3T3 fibroblasts significantly reduced the antitumor reactivity of the TDLN cells. We hypothesize that the local secretion of IFN-γ or IL-2 may have induced a significant allogeneic response to 3T3 determinants that suppressed the response to MCA 207.

DISCUSSION

We have demonstrated the utility of syngeneic or allogeneic fibroblasts modified to secrete GM-CSF to enhance immunity to a tumor inoculum in vivo. This was observed with either a weakly (i.e., MCA 207) or poorly (i.e., D5) immunogenic tumor and underscores the potential utility of this approach in treating diverse human cancers. Importantly, we found that syngeneic fibroblasts were superior to allogeneic fibroblasts as vehicles to deliver GM-CSF. In prior studies, we have found that the amount of GM-CSF elaborated locally correlated directly with the enhancement of immunity observed in the draining LNs. Hence, increasing the numbers of GM-CSF-secreting fibroblasts admixed with wild-type tumor cells should result in a corresponding increase in tumor immunity. This was observed with syngeneic fibroblasts but not with allogeneic cells. At high fibroblast: tumor cell ratios, allogeneic fibroblasts resulted in suppressed immune responses in the draining LNs.

As would be expected, the use of increasing numbers of allogeneic fibroblasts within the tumor inoculum elicited an immune response against alloantigens. This was observed in the draining LNs in which we could document immunologically specific cytolytic reactivity and cytokine release by lymphoid cells in response to allogeneic targets. However, the more critical response that was altered by the presence of alloantigen was the immune response to tumor cells. We observed at the highest allogeneic fibroblast:tumor ratio a decreased amount of IFN-γ and GM-CSF released by draining LNs in response to tumor cells with a concomitant increased release of IL-10. We hypothesize that this alteration of cytokines released by the draining LN cells reflects a shift from a type 1 response (i.e., IFN-γ and GM-CSF) to a type 2 response (i.e., IL-10), which was reflected by a suppressed antitumor response. In previous studies, we have shown that the neutralization of IFN-γ and GM-CSF by mAbs after adoptive transfer...
of immune LN cells abrogated tumor regression, whereas the neutralization of IL-10 in the same model augmented antitumor immunity mediated by the transferred cells (25). In those studies, we have documented that the growth of MCA 207 tumor resulted in the induction of five dominant Vβ T-cell subpopulations with different immunological reactivity to MCA 207 antigen. Using mAbs reactive to these five dominant Vβ T-cell subsets (Vβ3, -5, -7, -8, and -11), we were able to isolate these subpopulations and assess the effect of in vivo priming with allogeneic fibroblasts. Anti-Vβ mAbs were used to activate LNs draining MCA 207 or MCA 207 admixed with GM-CSF-secreting fibroblasts. After in vitro culture, a relatively homogeneous population of T cells specific for the anti-Vβ mAbs (>90%) used for activation was obtained. In our previous study, we have shown that Vβ8+ cells were a major T-cell subpopulation involved in mediating tumor regression in this model (25). The presence of allogeneic fibroblasts was associated with a dramatic decrease in IFN-γ and GM-CSF released by Vβ8+ cells in response to tumor antigen and increased IL-10 elaboration. In addition, Vβ5+ and Vβ7+ cells were induced to secrete significantly greater amounts of IL-10 in response to tumor antigen, with a concomitant decrease in secretion of IFN-γ and GM-CSF. The cytokine patterns released by these lymphoid populations in response to 3T3 target cells was similar to the response observed with tumor targets.

In our animal model, we noted that the admixture of MCA 207 with IL-2 or IFN-γ-secreting allogeneic fibroblasts suppressed the induction of immune draining LN cells. We have reported previously that D5 tumor cells modified genetically to secrete IL-2 or IFN-γ when inoculated into the flank of B6 mice did not result in the induction of enhanced immunity of draining LN cells, although these tumor cells exhibited reduced tumorigenicity (13). We hypothesize that the secretion of IL-2 or IFN-γ by allogeneic fibroblasts most likely enhanced the immune response to the alloantigens expressed by the fibroblasts. It is well known that the elaboration of IFN-γ up-regulates the expression of MHC molecules on cells transduced to secrete the transgenic protein. In addition, the in vivo administration of IL-2 has been shown to enhance the host response to immunization with alloantigens (26). The generation of a strong alloresponse appears to suppress the induction of tumor immunity in our model.

In summary, there has been significant interest in the use of cytokine-secreting fibroblasts as adjuvant reagents in tumor vaccines. Fibroblasts are useful cellular reagents to enhance the immunogenicity of tumor cell vaccines. However, the use of allogeneic fibroblasts can be associated with suppressed immune responses when used in high fibroblastofern cell ratios and should be avoided in clinical settings.

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Reduced Efficacy of Allogeneic *versus* Syngeneic Fibroblasts Modified to Secrete Cytokines as a Tumor Vaccine Adjuvant

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