Award Number:  DAMD17-01-1-0429

TITLE:  Analysis of Tumor Antigen-Specific Tc1 and Tc2 CD8 Effector Cell Subpopulations as Potential Therapeutics Agents in the Treatment of Progressive Breast

PRINCIPAL INVESTIGATOR:  Mark J. Dobrzanski, Ph.D.

CONTRACTING ORGANIZATION:  Trudeau Institute, Incorporated Saranac Lake New York 12983

REPORT DATE:  September 2004

TYPE OF REPORT:  Final

PREPARED FOR:  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:  Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Analysis of Tumor Antigen-Specific Tc1 and Tc2 CD8 Effector Cell Subpopulations as Potential Therapeutics Agents in the Treatment of Progressive Breast Cancer

Mark J. Dobrzanski, Ph.D.

Trudeau Institute, Incorporated
Saranac Lake New York 12983
E-Mail: mdobrzanski@trudeauinstitute.org

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

Approved for Public Release; Distribution Unlimited

Cytokines, type 1/Type 2 Immune Response, interferon-γ, chemotherapy, co-therapy

Unclassified

Unclassified

Unclassified

Unclassified
Table of Contents

Cover.........................................................................................................................
SF 298.........................................................................................................................
Table of Contents...........................................................................................................
Introduction................................................................................................................1
Body...............................................................................................................................1
Key Research Accomplishments..................................................................................3
Reportable Outcomes.................................................................................................4
Conclusions................................................................................................................4
References..................................................................................................................4
Appendices................................................................................................................5
INTRODUCTION
Adoptive CD8 T cell immunotherapy has been shown to be a viable modality for the treatment of certain human cancers (1-4). Aside from their direct cytolytic potential, CD8 T cells can be further classified into two distinct effector cell types based on their cytokine-secreting profiles following tumor antigen encounter. Type 1 CD8 T cells (Tc1) produce IFN-γ whereas, Type 2 CD8 T cells (Tc2) secrete IL-4, IL-5, IL-10 and GM-CSF. Such cytokines can not only have diverse inhibitory effects on tumor cells themselves, but also affect the nature of the immune response toward progressively growing malignancy. Although the existence of Tc1 and Tc2 effector cells have been demonstrated in patients with various clinical conditions (2), the nature and regulatory roles of these T cell subpopulations in breast cancer and immunotherapy remain unclear. It is conceivable that these "multifunctional" subpopulations can effectively regulate breast tumor growth and dissemination by different mechanisms. More importantly, since many breast cancers demonstrate considerable heterogeneity in the clinical course of their disease, treatment with tumor-specific immunotherapeutic agents that potentially afford multiple and diverse mechanisms of tumor eradication may enhance therapeutic benefits and provide a more favorable clinical outcome. It is our contention that either tumor-reactive Tc1 or Tc2 cells can initiate host-mediated antitumor responses and thus act as potential cellular vaccines. Moreover, such cellular therapies can not only act as primary therapeutic agents but also be effective as adjuvant therapies with other conventional clinical treatments such as chemotherapy.

BODY
The original Aims of the program remained unchanged. In the first year (Aim 1), we had developed and characterized a murine breast cancer model that correlated with defined TNM breast cancer classification stages of clinically progressive disease. We generated an aggressive mammary adenocarcinoma cell line, expressing a surrogate tumor antigen hemaagglutinin (TSA-HA), that was used to evaluate the therapeutic efficiency of adoptively transferred tumor antigen-specific Tc1 or Tc2 cells in mice with defined clinical stages of breast cancer based on anatomic tumor measurement and dissemination. In Aim 2, our studies addressed: (i) can adoptive immunotherapy with functionally-distinct tumor-antigen specific Tc1 or Tc2 effector cell subpopulations be an effective therapeutic agent in breast cancer and (ii) provide insight on their potential mechanisms that initiate and/or influence recipient antitumor immune responses at various stages of breast malignancy. In Aim 3, we investigated the use of two clinically-relevant cyto-reductive drugs (i.e. methotrexate (MTX) and 5-Fluorouracil (5-FU)) as potential co-therapeutic agents with CD8 effector cell therapy in progressive breast cancer. A number of the experiments proposed in Aim 3 have been achieved or are near completion.

Characterization of the TSA-HA tumor cell line and murine breast cancer therapy model.
Tumor cell lines of TSA-HA were generated by transfecting the TSA breast adenocarcinoma with influenza hemagglutinin (HA) cDNA and clones were selected by standard G418 selection methods. Flow cytometric analysis using specified cell surface monoclonal antibody detection of resulting tumor cell lines showed that nearly all TSA-HA tumor cells expressed cell surface HA antigen whereas parent TSA cells showed no detectable levels (5, 6). Moreover, both parent and TSA-HA tumor cell lines expressed MHC Class I and CD44 suggesting that the former retained a similar phenotype to that of later following transfection and selection procedures. In parallel studies we assessed potential differences in the tumor growth rates among parent and TSA-HA tumor cell lines in vivo. Both parent and TSA-HA tumor cells progressively grew in vivo without evidence of spontaneous regression when injected subcutaneously (1 x 10⁵ cells) into mammary fat pads of normal syngeneic BALB/c mice. Moreover, both tumor cell lines were observed to undergo spontaneous metastases to regional lymph nodes and lungs that were grossly evident by 18-25 days following tumor challenge (data not shown). Collectively, these studies establish and characterize, in part, a tumor model that correlates with both local and clinically disseminated stages of progressive breast cancer.

1.
Tumor regression by adoptively transferred Tc1 and Tc2 effector cells in mice with established malignancy.

To initially address the potential therapeutic role of cytokine-polarized tumor antigen-specific Tc1 and Tc2 effector T cell subpopulations in established breast cancer malignancies, we generated and characterized HA-specific T effector cell subpopulations in vitro from HA-T cell receptor transgenic mice (Aim 1 and Ref. 6). Following TSA-HA tumor challenge, graded numbers of in vitro-generated effector T cell subpopulations were intravenously transferred and therapeutic efficacy was evaluated by tumor volume and growth rate. As shown in Figure 1A, both Tc1 and the functionally-distinct Tc2 effector cell subpopulations effectively delayed orthotopic TSA-HA mammary tumor cell growth and mediated tumor regression in mice with established seven-day tumor. Concomitantly, we assessed the immunological specificity of HA-tumor antigen specific Tc1 and Tc2 effector cell therapy. As shown in Figure 1B, transfer of Tc1 and Tc2 effector cells into mice challenged with non-HA-expressing TSA parent line showed no detectable therapeutic effect on tumor growth or regression when compared to that of untreated control TSA tumor-bearing mice. Although both effector cell therapies appeared substantially effective and highly tumor antigen-specific in delaying mammary tumor cell growth they were non-the-less ineffective in establishing total long-term tumor eradication as tumor growth and progression among treated animals did not abate (Fig 1A). These studies suggest that tumor-reactive Tc1 or Tc2 effector cell subpopulations did effectively participate in T cell-mediated antitumor responses and adoptive immunotherapy however, with limited success in the treatment of breast cancer.

MTX and 5-FU enhance Tc2 effector cell therapy in the treatment of progressive breast cancer.

Since adoptive immunotherapy with either tumor-reactive Tc1 or Tc2 effector cell subpopulations as “single-treatment agents” appeared effective, yet limited, in preventing late stage tumor progression, we investigated the use of cytoreductive drugs as potential co-therapeutic agents to adoptively transferred CD8 effector cells. Using our TSA-HA immunotherapy tumor model, we treated tumor-bearing mice with single dose chemotherapy (day 7 post tumor challenge) using either MTX or 5-FU. Three days later, select groups of mice received Tc2 adoptive immunotherapy and tumor volumes and growth rates were monitored. As shown in Fig. 2, treatment with 5-FU alone (50 mg/kg body weight) effectively delayed tumor growth when compared to groups of untreated control tumor-bearing mice. However, when combined with Tc2 effector cell therapy, both tumor growth rates and volumes were markedly diminished when compared to groups of animals receiving either chemotherapy alone, effector cell therapy alone or nothing. This suggested that pretreatment of tumor-bearing mice with 5-FU chemotherapeutic agents are synergistic and enhanced the therapeutic efficacy of Tc2 effector cell-mediated immunotherapy. Moreover, when groups of mice were treated with either high (500 mg/kg body weight) or low (5 mg/kg body weight) doses of 5-FU prior to effector cell therapy, our studies showed that both enhanced therapeutic responses and treatment survival were markedly lower than that achieved in mice at the 50 mg/kg dose (Table 1). Similar results were obtained in corresponding studies using MTX (Fig 3 and Table 2).

Co-therapy with MTX, but not 5-FU, augments Tc1 effector cell immunotherapy in progressive breast cancer.

Since 5-FU and MTX have been previously shown to differ in both their pharmacokinetics, mechanisms of action, and metabolism/catabolism in vivo (7, 8), we investigated their co-therapeutic effects on functionally-distinct Tc1 effector cells. Seven days following tumor challenge, mice were treated intraperitoneally with single dose chemotherapy using either MTX (50 mg/kg body weight) or 5-FU (50 mg/kg body weight). Three days later, select groups of mice received Tc1 effector cell therapy and tumor growth rates and volumes were determined. As shown in Table 3, treatment with 5-FU alone effectively delayed tumor growth when compared to groups of untreated control mice. However, when combined with Tc1 effector cell therapy, tumor growth rates and volumes were similar to groups
receiving Tc1 therapy alone. However, in corresponding groups of mice receiving MTX and Tc1 co-therapy, both tumor growth rate and volumes were markedly reduced when compared to that of animals receiving either chemotherapy alone, effector cell therapy alone or nothing. This suggested that pre-treatment of tumor-bearing mice with MTX, and not 5-FU, chemotherapeutic agents appeared synergistic and markedly enhanced the therapeutic efficacy of Tc1 effector cell-mediated immunotherapy.

KEY RESEARCH DEVELOPMENTS (Project Summary):

- Successful generation of a TSA-HA tumor cell line expressing hemagglutinin (HA) as a surrogate tumor-associated antigen by transfection with influenza hemagglutinin (HA) cDNA.
- Phenotypic characterization of the TSA-HA tumor cell line shows similarly high levels of surface MHC Class I and CD44 when compared to parent TSA cell line in vitro.
- TSA-HA tumor cells show similar growth kinetic when compared to TSA parent line in vivo.
- TSA-HA mammary tumor cells progressively grow in vivo without evidence of spontaneous regression when injected orthotopically into the mammary fat pads.
- Orthotopically injected TSA-HA mammary tumor cells undergo spontaneous metastases to lymph nodes and systemic organs that is grossly evident between days 18 and 24 post tumor challenge. Thus providing a breast tumor model that correlates with defined TNM breast cancer classification stages of clinically progressive disease.
- Generation and characterization of HA antigen-specific Tc1 and Tc2 CD8 effector T cells from HA-TcR transgenic mice (completed in Aim 1 and Ref 6).

Aim 2. Assessment of Tc1 and Tc2 effector cell therapy in mice with different stages of disease progression.

- Single-dose adoptive transfer of functionally-distinct Tc1 and Tc2 effector cell subpopulations effectively delayed tumor growth and mediated tumor regression in mice with established TSA-HA tumor development.
- However, Tc2 effector cell therapies appeared less effective than Tc1 in mice with similar tumor burdens.
- Flow cytometric analysis showed that adoptively transferred Tc1 and Tc2 cells localized in appreciable numbers at the site of tumor growth.
- Effector Tc1 and Tc2 cell migration and antitumor effects were tumor antigen-specific.
- Tc1 effector cell therapy was titratable and highly dependent on effector cell-derived IFN-γ.

Aim 3. Conventional chemotherapeutic agents used in the current treatment of breast cancer patients enhance the therapeutic effectiveness of "functionally-distinct" Tc1 and Tc2 effector cell therapies in animals with progressive breast cancer.

- Co-therapeutic effects by MTX and 5-FU were dependent on drug dose, where both co-therapeutic responses and treatment survival and tolerance were achieved at doses of 50 mg/kg total body weight.
- Single dose treatments with either methotrexate (MTX) or 5-Fluorouracil (5-FU) chemotherapeutic agents markedly enhanced therapeutic effects of Tc2 effector cell-mediated immunotherapy and delayed mammary tumor cell growth in mice with established malignancy.
- MTX, but not 5-FU, acted synergistically with Tc1 effector cell immunotherapy by delaying tumor cell growth rates and volumes in mice with progressive breast cancer.
REPORTABLE OUTCOMES:

- Generation of an HA-Transfected TSA Mammary Adenocarcinoma Cell Line (TSA-HA).

CONCLUSIONS

The studies, to date, in this proposal includes the development and characterization of a murine breast cancer model designed to evaluate select tumor antigen-specific T cell subpopulation responses and their potential role in adoptive T cell immunotherapy. Our previous results suggest that both Tc1 and Tc2 effector cell subpopulations significantly participate in T cell-mediated antitumor responses and play a potential role in cellular adoptive immunotherapy for the treatment of progressive breast malignancies. Specifically, we relate (i) that substantial numbers of systemically transferred Tc1 or Tc2 effector cell subpopulations preferentially accumulated at the site of tumor growth, (ii) single dose treatment with either tumor-reactive Tc1 or Tc2 cells induced both tumor regression and delay in the rates of tumor growth in mice with established breast cancer, (iii) antitumor responses by adoptively transferred Tc1 effector cells were highly tumor antigen-specific and appeared to be highly dependent on donor cell-derived IFN-γ. In the final aim of this project, we assessed the effectiveness of Tc1 or Tc2 effector cell therapies as potential adjuvants to current clinical therapeutic modalities such as chemotherapy. Use of single dose MTX or 5-FU as “first-line” cancer treatments acted synergistically with adoptively transferred Tc1 or Tc2 effector cells. Although CD8 effector cell therapies were markedly enhanced in combination with such chemotherapeutic agents by delaying tumor cell growth and volumes, treatments appeared non-the-less ineffective in establishing total long-term tumor eradication to progressive breast malignancy. It is our contention that the therapeutic efficiency by adoptively transferred T cells can be enhanced with combinatorial approaches that include either current experimental or standard clinical therapeutic strategies. With the current capacity to isolate tumor-antigen specific T cells from cancer patients (1), ex vivo generation, propagation and re-infusion of functionally-distinct Tc1 or Tc2-like effector cells may offer an alternative and/or new strategy for successful tumor immunotherapy and vaccine development for patients with breast cancer. Lastly, our studies suggest a significant role for Tc1 and Tc2 effector cell subpopulations in T cell-mediated antitumor responses and therapy and merit further investigation as potentially clinically-relevant anticancer therapies for breast cancer.

REFERENCES:


**APPENDICES (Aim 3 studies only):**

*Figure 1.* Therapeutic efficacy and specificity of adoptive transferred Tc1 or Tc2 effector cells in mice with established mammary tumors.

*Figure 2.* Synergistic effects of single dose 5-FU on tumor-specific Tc2 effector cell therapy in mice with progressive breast cancer.

*Figure 3.* Synergistic effects of MTX on tumor-specific Tc2 effector cell therapy in mice with progressive breast cancer.

*Table 1.* Therapeutic effects of high and low drug doses of 5-FU on tumor-specific Tc2 effector T cell therapy of progressive breast cancer.

*Table 2.* Therapeutic effects of high and low drug doses of MTX on tumor-specific Tc2 effector T cell therapy of progressive breast cancer.

*Table 3.* Comparative effects of MTX and 5-FU on Tc1 effector cell therapy among animals with progressive breast cancer.
Figure 1. Therapeutic efficacy and specificity of adoptive transferred Tc1 or Tc2 effector cells in mice with established mammary tumors. Syngeneic mice (n=6/gp) were injected subcutaneously (sc) in the mammary fat pad region with $1 \times 10^5$ TSA-HA tumor cells. Seven days later, tumor-reactive Tc1 or Tc2 effector cells were adoptively transferred into tumor-bearing mice and tumor volumes (A) were determined. In B, mice (n=6/gp) were injected with $1 \times 10^5$ non-HA expressing TSA parental cells. Seven days later, tumor-reactive Tc1 or Tc2 effector cells were adoptively transferred into tumor-bearing mice and tumor volumes (A) were determined. Tumor volumes were measured using vernier calipers and volumes obtained by multiplying the measured length by the measured width by the calculated mean of these measured values. Results are representative of five independent experiments.
Synergistic effects of single dose 5-FU on tumor-specific Tc2 effector cell therapy in mice with progressive breast cancer. Mice were injected sc in the mammary fat pad region with $1 \times 10^6$ TSA-HA tumor cells. Seven days later, groups of mice were treated with either 5-FU (50 mg/kg body weight) alone or 5-FU and $1 \times 10^7$ Tc2 effector cells at specified times and tumor volumes (left panel) and relative tumor growth rates (right panel) were determined. Untreated and Tc2 effector cell-treated tumor-bearing mice served as controls. At corresponding time intervals, tumor volumes were measured using vernier calipers and volumes obtained by multiplying the measured length by the measured width by the calculated mean of these measured values. The relative tumor growth rate is determined by regression analysis (95% confidence intervals). The absolute value of the slope of the regression line is the tumor growth rate. The ratio of the growth rates among groups of mice receiving different treatments to corresponding groups of untreated mice x 100 is depicted on the x-axis. Results are representative of three independent experiments.
Fig 3. Effects of MTX On Tc2 Effector Cell Therapy Of Animals with Progressive Breast Cancer

Synergistic effects of MTX on tumor-specific Tc2 effector cell therapy in mice with progressive breast cancer. Mice were injected subcutaneously in the mammary fat pad region with $1 \times 10^5$ TSA-HA tumor cells. Seven days later, groups of mice were treated with either MTX (50 mg/kg body weight) alone or MTX and $1 \times 10^7$ Tc2 effector cells at specified times and tumor volumes (left panel) and relative tumor growth rates (right panel) were determined. Untreated and Tc2 effector cell-treated tumor-bearing mice served as controls. Tumor volumes and growth rates were determined as described in Fig. 2. Results are representative of three independent experiments.
Table 1. Effects of 5-FU on Tc2 Effector Cell Therapy Among Animals With Breast Cancer

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Drug Dose (mg/kg)</th>
<th>Slope</th>
<th>Tumor Growth Rate (Percent)</th>
<th>Treatment Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>-1317.00</td>
<td>100.0</td>
<td></td>
<td>0% (0/18)</td>
</tr>
<tr>
<td>Tc2 Alone</td>
<td>-654.20</td>
<td>49.7</td>
<td></td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>5-FU Alone</td>
<td>5</td>
<td>-1157.00</td>
<td>87.9</td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>Tc2 + 5-FU</td>
<td>-575.20</td>
<td>43.7</td>
<td></td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>5-FU Alone</td>
<td>50.0</td>
<td>-726.00</td>
<td>55.1</td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>Tc2 + 5-FU</td>
<td>-306.00</td>
<td>23.2</td>
<td></td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>5-FU Alone</td>
<td>500.0</td>
<td>ND</td>
<td>ND</td>
<td>16% (2/12)</td>
</tr>
<tr>
<td>Tc2 + 5-FU</td>
<td></td>
<td></td>
<td></td>
<td>16% (2/12)</td>
</tr>
</tbody>
</table>

Therapeutic effects of high and low drug doses of 5-FU on tumor-specific Tc2 effector T cell therapy of progressive breast cancer. Mice (n=6-10/gp) were injected sc in the mammary fat pad region with 1 x 10^5 TSA-HA tumor cells. Seven days later, groups of mice were treated with various doses of 5-FU (500, 50 or 5 mg/kg body weight) alone or with corresponding doses of 5-FU and 1 x 10^7 Tc2 effector cells at specified times and the relative tumor growth rates were determined. Untreated and Tc2 effector cell-treated tumor-bearing mice served as controls. Tumor growth rates were determined as described in Fig. 2.
Table 2. Effects of MTX on Tc2 Effector Cell Therapy Among Animals With Breast Malignancy

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Drug Dose (mg/kg)</th>
<th>Slope</th>
<th>Tumor Growth Rate (Percent)</th>
<th>Treatment Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td>-1317.00</td>
<td>100.0</td>
<td>0% (0/18)</td>
</tr>
<tr>
<td>Tc2 Alone</td>
<td></td>
<td>-668.70</td>
<td>50.8</td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>MTX Alone</td>
<td>5</td>
<td>-825.00</td>
<td>62.6</td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>Tc2 + MTX</td>
<td></td>
<td>-514.20</td>
<td>39.0</td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>MTX Alone</td>
<td>50</td>
<td>-636</td>
<td>48.3</td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>Tc2 + MTX</td>
<td></td>
<td>-422.2</td>
<td>32.1</td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>MTX Alone</td>
<td>500.0</td>
<td>-595.00</td>
<td>45.2</td>
<td>50% (6/12)</td>
</tr>
<tr>
<td>Tc2 + MTX</td>
<td></td>
<td>-648.60</td>
<td>49.2</td>
<td>17% (2/12)</td>
</tr>
</tbody>
</table>

Therapeutic effects of high and low drug doses of MTX on tumor-specific Tc2 effector T cell therapy of progressive breast cancer. Mice (n=6-10/gp) were injected subcutaneously in the mammary fat pad region with 1 x 10^6 TSA-HA tumor cells. Seven days later, groups of mice were treated with various doses of MTX (500, 50 or 5 mg/kg body weight) alone or corresponding doses of 5-FU and 1 x 10^7 Tc2 effector cells at specified times and the relative tumor growth rates were determined. Untreated and Tc2 effector cell-treated tumor-bearing mice served as controls. Tumor growth rates were determined as described in Fig. 2.
Table 3. Comparative Effects of Different Chemotherapeutic Agents on Tc1 Effector Cell Therapy Among Animals With Breast Malignancy

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Drug Dose (mg/kg)</th>
<th>Slope</th>
<th>Tumor Growth Rate (Percent)</th>
<th>Treatment Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td>-1317.00</td>
<td>100.0</td>
<td>0% (0/18)</td>
</tr>
<tr>
<td>Tc1 Alone</td>
<td></td>
<td>-619.30</td>
<td>47.0</td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>5-FU Alone</td>
<td>50.0</td>
<td>-726.00</td>
<td>55.1</td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>Tc1 + 5-FU</td>
<td></td>
<td>-589.20</td>
<td>44.7</td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>MTX Alone</td>
<td>50.0</td>
<td>-595.00</td>
<td>45.2</td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>Tc1 + MTX</td>
<td></td>
<td>-359.70</td>
<td>27.3</td>
<td>100% (18/18)</td>
</tr>
</tbody>
</table>

Therapeutic effects of MTX or 5-FU on tumor-specific Tc1 effector T cell therapy of progressive breast cancer. Mice (n=6-10 mice/gp) were injected sc in the mammary fat pad region with $1 \times 10^5$ TSA-HA tumor cells. Seven days later, groups of mice were treated with either MTX alone (50 mg/kg body weight), 5-FU alone (50 mg/kg body weight) or corresponding chemotherapeutic agents and $1 \times 10^7$ Tc1 effector cells at specified times and the relative tumor growth rates were determined. Untreated and Tc1 effector cell-treated tumor-bearing mice served as controls. Tumor growth rates were determined as described in Fig. 2.