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

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**Title and Subtitle:**
Analysis of Tumor Antigen-Specific Tc1 and Tc2 CD8 Effector Cell Subpopulations as Potential Therapeutic Agents in the Treatment of Progressive Breast Cancer

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**Abstract:**
Cytolytic CD8 T cells fall into two subpopulations based on cytokine-secretion. Type 1 CD8 cells (Tc1) characteristically secrete IFN-γ, whereas type 2 CD8 cells (Tc2) secrete IL-4 and IL-5. Using an aggressive TSA mammary adenocarcinoma cell line, expression HA as a surrogate tumor-associated antigen, we assessed the relative therapeutic effects of adoptively transferred HA tumor antigen-specific Tc1 and Tc2 CD8 effector cells in tumor-bearing mice at different stages of malignancy. Tc1, but not Tc2, subpopulations effectively delayed tumor cell growth and mediated tumor regression in mice with early (Day 7) stages of established tumor development. However, neither therapy was effective in more advanced (Day 21) stages of malignancy. Flow cytometric analysis showed that donor Tc1 cells accumulated at the tumor site and antitumor effects were highly tumor antigen-specific. Titration studies of Tc1 effector cells showed that protection and therapy were dose-dependent in vivo. Tc1 effector cells derived from IFN-γ-deficient mice were less therapeutically effective than that of corresponding wild-type mice suggesting that effector cell-derived IFN-γplayed a significant role in Tc1-subpopulations in adoptive immunotherapy for the treatment for the treatment of breast cancer.
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INTRODUCTION
Adoptive CD8 T cell immunotherapy has been shown to be a viable modality for the treatment of certain human cancers (1). 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. Such cellular therapies can not only act as primary therapeutic agents but also be effective as adjuvant therapies among patients that do not tolerate or are non-responsive to conventional clinical treatments.

BODY
The original Aims of the program after its second year remain 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 will be used to evaluate the therapeutic efficiency of tumor antigen-specific T cell adoptive immunotherapy in mice with defined clinical stages of breast cancer based on anatomic tumor measurement and dissemination. In Aim 2, our studies will address: (i) can adoptive immunotherapy with tumor-antigen specific Tc1 and 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. A number of the experiments proposed in Aim 2 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. 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. As shown in Figure 2, both parent and TSA-HA tumor cells progressively grew in vivo without evidence of spontaneous regression when injected subcutaneously (1 x 10^3 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.
Tumor regression by adoptively transferred Tc1, but not Tc2, effector cells in mice with various stages of established malignancy.

To initially address the potential therapeutic role of cytokine-polarized tumor antigen-specific Tc1 and Tc2 effector T cell subpopulations in either localized or disseminated 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). At different time intervals following TSA-HA tumor challenge, graded numbers of in vitro-generated effector T cell subpopulations will be intravenously transferred and therapeutic efficacy will be evaluated by tumor growth rate and survival. As shown in Figure 3A, Tc1, but not Tc2, effector cell subpopulations effectively delayed orthotopic TSA-HA mammary tumor 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 3B, transfer of Tc1 and Tc2 effector cells into mice challenged with non-HA-expressing TSA parent line showed no detectable therapeutic effect in tumor growth or regression when compared to that of untreated control TSA tumor-bearing mice. Moreover, neither therapy was effective in mice exhibiting more advanced (>21 days) stages of either spontaneous (subcutaneous local/regional tumor challenge) or experimental (intravenously-induced tumor metastases) malignancy (Fig. 4A and B, respectively). This suggests that adoptively transferred Tc1 effector cell therapy is not only tumor antigen specific, but also dependent, in part, on tumor location, maturation and stage of development and progression.

Localization and antitumor effects of adoptively transferred HA-specific Tc1 effector cells to sites of tumor growth are tumor-antigen specific.

Using multicolor flow cytometric analyses and congenic mice to distinguish between donor and tumor-bearing recipient cell populations (3), we investigated whether adoptively transferred donor Tc1 effector cells localize and persist at the sites of local tumor growth over time. As shown in Figure 5, donor Tc1 cells accumulated at the tumor site among mice exhibiting seven-day established orthotopic tumors with peak levels at 21 days following therapy. In contrast, negligible proportions (<0.5%) of donor Tc1 cells were observed in corresponding mice receiving non-HA-expressing TSA parent tumor cell challenge at all time points tested. This suggested that Tc1 effector cells did accumulate and localize at sites of tumor growth and that such trafficking was highly tumor antigen-specific. In parallel studies, we evaluated the accumulation of recipient-derived mononuclear cells at the sites of tumor growth after Tc1 effector cell-mediated therapy. As shown in Figure 6A, absolute cell numbers of tumor-bearing recipient-derived mononuclear cell were elevated by day 21 following Tc1 effector cell therapy whereas corresponding cell populations among untreated control groups were comparatively lower. Concomitantly, Tc1 effector cell-treated mice had lower absolute tumor cell numbers whereas tumor cell numbers among untreated control groups were substantially higher at corresponding time points following tumor challenge (Fig. 6B). This suggests that therapeutic responses by adoptively transferred Tc1 effector cells were due, in part, to the recruitment and presence of recipient-derived immune cell populations that correlate with a decrease and/or delay in local tumor cell numbers and growth, respectively.

Role of effector cell-derived IFN-γ in Tc1-mediated therapy.

Since Tc1 cells produce substantial amounts of IFN-γ (2, 3) and therapeutic efficacy of adoptive immunotherapy is proportional to the numbers of transferred immune cells, we quantitatively analyzed the role and antitumor effects of Tc1 effector cell-derived IFN-γ. Briefly, different effector cell concentrations of Tc1 cells derived from either wildtype or IFN-γ-knockout HA-T cell receptor transgenic mice, were used to treat mice with established seven-day orthotopic tumors. As shown in Figure 7A, mice receiving doses of 50-100 x 10^5 of wildtype HA-specific effector cells showed a decrease in tumor growth and progression when compared with that of
untreated tumor-bearing control animals. Moreover, transfer of 10-fold less wildtype Tc1 effector cells, at numbers as low as $5 \times 10^5$, resulted in a similar therapeutic effect. In contrast, groups of mice receiving a similar dose of IFN-γ-deficient Tc1 cells showed no therapeutic effect and only when given a 20-fold higher effector cell number ($100 \times 10^5$) did animals start to show significant ($P < 0.05$) decreases in tumor cell growth (Fig 7B). These results show that, on a per cell basis, Tc1 effector cell-derived IFN-γ played a significant role in Tc1-mediated tumor therapy.

**KEY RESEARCH ACCOMPLISHMENTS**


- 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*).

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

- Single-dose adoptive transfer of Tc1, but not Tc2, effector cell subpopulations effectively delayed tumor growth and mediated tumor regression in mice with early stages of established TSA-HA tumor development.
- However, both Tc1 and Tc2 effector cell therapies were much less effective in mice with elevated tumor burdens and more advanced stages of tumor cell growth.
- Flow cytometric analysis showed that adoptively transferred Tc1 cells localized in appreciable numbers at the site of tumor growth.
- Effector cell migration and antitumor effects were tumor antigen-specific.
- Tc1 effector cell therapy was titratable and highly dependent on effector cell-derived IFN-γ.

**REPORTABLE OUTCOMES**

- HA-Transfected TSA Mammary Adenocarcinoma Cell Line (TSA-HA).
CONCLUSIONS
These studies performed in year two of 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 results suggest that Tc1 effector cell subpopulations play a significant role in T cell-mediated therapy and play a potential role in adoptive immunotherapy for the treatment of breast cancer. Specifically, we relate (i) that substantial numbers of systemically transferred Tc1 effector cell populations preferentially accumulated at the site of tumor growth, (ii) single dose treatment with tumor-reactive Tc1 cells induced tumor regression in mice with established breast cancer, (iii) systemic antitumor responses by adoptively transferred Tc1 cells were highly tumor antigen-specific and appeared to be highly dependent on donor cell-derived IFN-γ, (iv) the level of Tc1-mediated therapy appeared to be influenced by the stage of tumor development in the breast. In the final aim of this project, we will assess the effectiveness of Tc1 effector cell therapy as a potential adjuvant to current clinical therapeutic modalities such as surgery, radiation and chemotherapy. 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 Tc1-like effector cells may offer an alternative and/or new strategy for successful tumor immunotherapy and vaccine development for patients with breast cancer.

REFERENCES:

APPENDICES:
Figure 1. Characterization of TSA and TSA-HA tumor cell surface antigen expression in vitro.
Figure 2. TSA and TSA-HA tumor cell growth kinetics among normal syngeneic mice in vivo.
Figure 3. Therapeutic effects and specificity of tumor antigen-specific Tc1 and Tc2 effector cell therapy in mice with established TSA-HA (A) or TSA (B) local/regional breast cancer.
Figure 4A. Therapeutic efficacy of adoptively transferred Tc1 effector cells at late stages of TSA-HA tumor maturation and progression.

Figure 4B. Effects of tumor antigen-specific Tc1 and Tc2 effector cell therapy in mice with established “late-stage” experimental metastases.

Figure 5. Select localization of adoptively transferred HA-tumor antigen-specific Tc1 effector cells in orthotopic tumors of mice with either TSA or TSA-HA established breast cancer.

Figure 6. Recipient-derived mononuclear (A) and tumor (B) cell numbers and kinetics in mammary tumors of mice with established breast cancer following antigen-specific Tc1 effector cell therapy.

Figure 7. Role of effector cell-derived IFN-γ in Tc1 effector cell-mediated therapy of mice with established mammary tumor.
Figure 2
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Tumor Growth Kinetics Among Wildtype BALB/c

- □ HA-TSA Cell Line
- ○ TSA Parent Cell Line

Gross Metastases to Draining LN

Days Post Tumor Challenge

Tumor Volume (mm³)
A. Therapeutic Effects of HA-Specific Effector Cell Therapy on Progressive TSA-HA Breast Cancer

B. Effects of HA-Specific Effector Cell Therapy on Progressive TSA Parent Line
Therapeutic Efficacy of Tc1 Effector Cell Therapy at Late Stages of TSA-HA Tumor Progression

Days Post Tumor Challenge (SC)

Effect of Effector Cell Therapy on Tumor Volume
Effects of T Cell Therapy Following Intravenous Challenge Of HA-Expressing TSA Tumor Cells

![Graph showing percent survival over days post tumor challenge](image)
Localization of Donor Tumor-Antigen-Specific Tc1 Effector Cells (Thy 1.2/CD8) in Tumors of Mice With Established Breast Cancer

_days Post Therapy_

<table>
<thead>
<tr>
<th>Days Post Therapy</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
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<tr>
<td>TSA-HA Tumor</td>
<td>2.7%</td>
<td>2.8%</td>
<td>4.4%</td>
<td>0.9%</td>
</tr>
<tr>
<td>TSA Parent Tumor</td>
<td>0.5%</td>
<td>0.0%</td>
<td>0.2%</td>
<td>0.2%</td>
</tr>
</tbody>
</table>

Thy 1.2

CD8
A. Tumor Infiltrating Mononuclear Cell Kinetics

- HA-Tc1 Effector Cell Therapy
- Untreated

B. TSA-HA Tumor Cell Growth Kinetics

- HA-Tc1 Effector Cell Therapy
- Untreated
A. Wild-Type HA-Tc1 Effector Cell Therapy

B. IFN-γ Deficient HA-Tc1 Effector Cell Therapy