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CONTRACTING ORGANIZATION: Providence Portland Medical Center Portland, Oregon 97213-2967

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13. **ABSTRACT (Maximum 200 Words)**

   When vaccination fails to protect the host from a subsequent challenge with a tumor, that tumor is generally characterized as nonimmunogenic. This designation suggests that the host has not recognized, or is tolerant of the tumor antigens. Our recent studies suggest that this is not true. We have demonstrated that progressively growing subcutaneous tumors sensitize tumor-specific T cells; however, the antigen-reactive T cells are polarized to secrete type 2 (T2) cytokines (e.g. IL-4 and IL-10), and lack therapeutic activity upon adoptive transfer. Conversely, immunogenic tumors induce predominantly type 1 (T1) antitumor responses, exhibiting highly polarized tumor-specific IFN-γ secretion. This proposal examines issues that are critical to understanding the mechanism for tumor regression following vaccination/immunotherapy. The first issue is whether tumor-specific T2 T cells, induced by progressively growing tumor, can inhibit the therapeutic efficacy of tumor-specific T1 T cells in our 4T1 mammary tumor model. If T2 T cells can inhibit therapeutic T cells it offers an explanation for the failure of tumor vaccine strategies and a possible approach to circumvent the inhibitory effect of T2 T cells. Aim 2 will test whether promoting a T1 response to vaccination will convert the nonimmunogenic 4T1 mammary tumor into a therapeutic vaccine.

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INTRODUCTION:

This proposal addresses several issues that are critical to understanding the mechanism for tumor regression following vaccination/immunotherapy. The first issue is whether tumor-specific T2 T cells, induced by progressively growing tumor, can inhibit the therapeutic efficacy of tumor-specific T1 T cells in our 4T1 mammary tumor model. If T2 T cells can inhibit therapeutic T cells it offers a possible explanation for the failure of tumor vaccine strategies in both non immunogenic animal models and patients with cancer. It also offers a possible approach to circumvent the inhibitory effect of T2 T cells. Aim 2 will test whether promoting a T1 response to vaccination will convert the nonimmunogenic 4T1 mammary tumor into a therapeutic vaccine. Successful completion of these studies will provide insight into whether it might be beneficial to polarize a tumor-specific or HER-2/neu-specific T1 cytokine response in breast cancer patients. If these preclinical studies document a significant therapeutic advantage it could lead to the initiation of a new clinical trial for women with breast cancer.
BODY:

Hypothesis/Purpose:
It is our hypothesis that lymphocytes in lymph nodes draining therapeutic vaccines exhibit superior anti-tumor activity because the tumor-specific T cells contained within produce T1 cytokines which mediate tumor regression. In contrast, nontherapeutic T cells produce T2 cytokines which do not contribute to tumor destruction and may interfere with the antitumor response. Our purpose is to test this hypothesis in the 4T1 mammary tumor model and evaluate the therapeutic efficacy of strategies that drive the development of a tumor-specific T1 cytokine response.

Technical Objectives:
Our preliminary studies suggested that we could generate T cells polarized to secrete T1 or T2 cytokines in response to stimulation with specific tumor cells. These studies also suggested that the therapeutic activity resides in the L-selectin<sup>Lo</sup> T cells that exhibit a T1 profile. The hypothesis of this objective is that a T1 cell mediates tumor regression through antigen-stimulated release of IFN-γ or other T1 cytokines, and that a tumor-specific T2 cell can inhibit this therapeutic activity by the secretion of T2 cytokines. To test this hypothesis, we proposed to first generate tumor-specific T1 and T2 T cells and test their antitumor efficacy against 3-day established pulmonary metastases. We will then be able to determine whether the addition of tumor-specific T2 T cells can inhibit the antitumor activity of T1 T cells.

1) To generate tumor-specific T1 and T2 T cells and test their antitumor efficacy against 3-day established pulmonary metastases

Initial studies with the 4T1 parental line proved to be highly variable. It became clear that in order to perform this study we would need to further optimize our vaccine strategy. Since the 4T1 tumor is a poorly immunogenic tumor it does not sensitize tumor-specific T cells efficiently. In order to improve priming of tumor specific T cells we decided to transduce the 4T1 breast cancer cell line with the gene encoding GM-CSF. Our recent report documents that this is an efficient method to prime tumor-specific T cells in the B16BL6 melanoma tumor model (Hu et al. in press J. Immunology, Oct. 15, 2000). Because 4T1 is heterogeneous in respect to both morphology and phenotype (Figure 1), it was cloned prior to being transduced. Figures 2 and 3 present data for 2 different 4T1 clones. These 2 clones of 4T1 are strikingly different in their expression of immunologically relevant molecules. The 4T1-7 clone expresses high levels of CD40 and ICAM-1 which should augment its efficacy as a vaccine.

Yet vaccination with this cell line did not provide increased protection over vaccination with the parental cell line (data not shown). Another clone, 4T1-9, did not express substantial amounts of either molecule and was also poorly immunogenic when used as a vaccine. Both of these clones appeared to be morphologically distinct, with 4T1-7 being highly adherent and the 4T1-9 being loosely adherent (see pictoral inserts in figures 2 and 3). (These 2 cell lines have been provided to Dr. James Mule's laboratory, at the University of Michigan, in a collaborative study to examine the role for CD40 in dendritic cell-based vaccine strategies.)

The 4T1-9 clone was transduced with the gene encoding GM-CSF, cloned by limiting dilution and clones screened for production of GM-CSF. One clone, 4T1-E10-9 (E10-9), secreting 15 ng/10<sup>6</sup> tumor cells/24 hours was selected for subsequent vaccine studies (data not shown). Briefly, mice were inoculated bilaterally s.c. with 10<sup>6</sup> 4T1- E10-9 tumor cells and Day 7 TVDLN harvested and L-selectin<sup>Lo</sup> T cells isolated using anti-L-Selectin (CD62) magnetic beads (Miltenyi Inc). L-selectin<sup>Lo</sup> T cells were activated with anti-CD3 (2c11) and expanded in IL-2. A summary of three independent cytokine release assays is shown in Figure 4. These results demonstrate that the E10-9 tumor cell vaccine is more effective at priming T cells that secrete IFN-γ in response to stimulation with specific tumor (4T1) but not the syngeneic but unrelated EMT-6 tumor cell. We then characterized the cells that mediated tumor-specific IFN-γ secretion. Effector T cells were separated into CD4+ or CD8+ T cells and stimulated with 4T1 or EMT-6. As a control T cells were stimulated with immobilized anti-CD3 (positive control) or nothing (negative control). The results of this experiment demonstrate that the tumor-specific IFN-γ is secreted by a CD8+ T cell (figure 5). These T cells were then adoptively transferred into mice with 3 day established 4T1 pulmonary metastases. Ten days later mice were killed,
their lungs harvested, insufflated with India ink and fixed. The number of pulmonary metastases was counted in a blinded fashion. Representative photographs of lungs for each group are shown in table I. This experiment clearly shows that the GM-CSF secreting tumor vaccine is significantly (p<0.05) more effective at priming TVDLN T cells with therapeutic activity.

Since our objective was to block IL-4 in order to generate tumor-specific type 1 effector cells, we vaccinated IL-4 knock-out (IL-4ko) mice. While effector T cells from mice did not secrete IL-4, they did not consistently generate T cells with therapeutic efficacy in the pulmonary metastases model (data not shown).

Another approach to block the effect of IL-4 is by using mice that cannot signal through their IL-4 receptor. STAT 6 knock-out (STAT 6 ko) mice lack the molecules required to transmit a signal from the IL-4 receptor. Thus, cells from these animals cannot respond to IL-4. We vaccinated STAT 6 ko mice, removed TVDLN 8 days later, isolated and activated the lymphocytes with anti-CD3 and expanded them in low dose IL-2 to generate effector T cells. This approach has provided us with tumor-specific effector T cells that are highly polarized to a type 1 cytokine profile (Figure 7), with no detectable secretion of tumor-specific IL-4 (data not shown). These highly polarized T cells have been effective at mediating significant tumor regression in 2 of 3 experiments. These results now provide us with two methods to generate tumor-specific T1 T cells. The first is by vaccinating animals and blocking IL-4 in vivo and in vitro, while providing IL-12. The second is by generating effector T cells in STAT-6 ko mice. As we prepare to examine whether the tumor-specific T2 effector T cells can block the therapeutic efficacy of tumor-specific type 1 effector T cells, having T1 effector T cells that either can (wt mice with cytokine manipulation) or cannot (STAT-6 ko) respond to the potential inhibitory activity of IL-4 will allow us to characterize further potential mechanisms of T cell regulation. Current studies are seeking to optimize the generation of tumor-specific T2 effector T cells to perform the second component of these studies.

Nota Bene:
An interesting observation has been that effector T cells generated from STAT-6 ko mice exhibit a highly polarized and extremely strong (25 ng/ml IFN-γ) type I cytokine response (Figure 7). However, it is not clear whether the response is truly tumor-specific, since the effector T cells also see the EMT-6 breast cancer cell line. EMT-6 is known to, like 4T1, express her2/neu, and since it is also a breast cancer it could share with 4T1 a spectrum of breast cancer antigens that may explain this observation. Current studies are aimed at repeating these studies with a syngeneic but unrelated colon or renal cancer cell line.

A particularly noteworthy and clinically relevant observation was that tumor-bearing animals that received highly polarized type 1 effector T cells exhibited substantial toxicity, including the death of some treated animals. Examination of serum from one animal at the time of severe toxicity revealed extraordinarily high levels of IFN-γ in the serum (> 30,000 pg/ml IFN-γ). We will continue to monitor the animals in these experiments carefully and seek additional support from a consulting pathologist as needed.
This page contains unpublished data that should be protected.

**KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research.

- Confirmation that L-selectin<sup>Lo</sup> TVDLN T cells contain the population with therapeutic activity (AAI 2000 abstract)
- Generation and characterization of 4T1 clones that exhibit strikingly different and immunologically interesting phenotypes
- STAT-6 ko mice vaccinated with a GM-CSF secreting 4T1 vaccine generate tumor-reactive T cells with capacity to secrete extraordinarily high levels (25 ng/ml/24 hrs) of IFN-γ.
- Adoptive transfer of tumor-reactive STAT-6 ko effector T cells is therapeutic.
REPORTABLE OUTCOMES:

- manuscripts, abstracts, presentations;
  
  Abstracts:
  
  S. Jensen, H-M. Hu, B.A. Fox  Priming Tumor Vaccine Draining Lymph Node T cells by GM-CSF Transduced 4T1. abstract # 55.6  FASEB/AAI, May 12-16, 2000
  
  presentations;
  
  None
  
  - patents and licenses applied for and/or issued;
  
  - None
  
  - degrees obtained that are supported by this award;
  
  - None
  
  - development of cell lines, tissue or serum repositories;
  
  - A series of 4T1 clones have been developed with distinctly different morphologic and phenotypic characteristics. Two of these cell lines have already been provided to Dr. J. Mule’ (University of Michigan) for use in a collaboration.
  
  - informatics such as databases and animal models, etc;
  
  - Characterization of the 4T1 clones is continuing. They may provide advantages over existing 4T1 model.
  
  - funding applied for based on work supported by this award;
  
  - None
  
  - employment or research opportunities applied for and/or received on experiences/training supported by this award.
  
  - None
CONCLUSIONS:

Our results suggest that using a GM-CSF transduced breast cancer vaccine increases the priming of tumor-specific T cells in the TVDLN, as determined by an increase in tumor-specific IFN-γ secretion in vitro and increased therapeutic efficacy in vivo. Furthermore, we have identified that downregulation of L-selectin expression as a marker for T cells with therapeutic potential is also true for this model of breast cancer (AAI 2000 Abstract and, Jensen et al. Manuscript in preparation). We have used these findings to optimize our vaccine model.

Using this GM-CSF secreting tumor vaccine model we have generated effector T cells from wild type (wt), IL-4 ko and STAT-6 ko mice. The vaccinated STAT-6 ko mice, deficient in IL-4 signalling, have consistently given us the most highly T1 polarized T cells. These will be critical for us to evaluate how tumor-specific T1 and T2 cells interact with each other. Furthermore, these reagents will help us determine whether tumor-specific T2 cells can inhibit the antitumor activity of tumor-specific T1 effector cells. The observation that infusion of large numbers of highly polarized T1 T cells from STAT-6 ko mice resulted in substantial toxicity and death of some animals suggests that we have reached a dose limiting toxicity with these highly polarized T cells. These findings will need to be considered as we begin to translate novel strategies to develop highly polarized tumor-specific T1 T cells for patients with breast cancer. While these findings are consistent with our hypothesis that a T1 cytokine response is critical for T cell-mediated tumor regression, it is puzzling why the STAT-6 ko T cells, which exhibit more than a log increase in IFN-γ secretion, don’t exhibit greater therapeutic efficacy in vivo. Our recent work in the B16BL6 melanoma model suggests that IFN-γ ko mice (GKO) can still generate effector T cells with therapeutic activity. These GKO effector cells don’t exhibit a T2 cytokine profile but do retain expression of LT-β, another T1 cytokine (Winter et al. submitted). Based on these observations in the C57BL/6 mouse model we plan to widen our characterization of the 4T1 BALB/c mouse model to include LT-β.

Related work in my laboratory is evaluating the immune response of patients on vaccine trials at our institution (melanoma, renal, breast and non small cell lung cancer). Of patients who had a tumor-specific in vitro response, 25% were T1 and 25% were T2. The other 50% exhibited a mixed T1 and T2 response to specific tumor (Meijer et al. abstract submitted). While these are preliminary studies and include only small numbers of patients, they suggest that a majority of patients studied (75%) had what we would consider an ineffective (T2 or mixed T1 and T2) immune response. The goal of this DOD proposal is to determine whether strategies that polarize the antitumor immune response in vivo / and or in vitro will augment the antitumor immune response to breast cancer. This goal is still an important one and may provide important insights that could be rapidly translated to augment existing immunotherapy strategies for patients with breast cancer or other malignancies.
REFERENCES:


S. Jensen, H-M. Hu, B.A. Fox  Priming Tumor Vaccine Draining Lmph Node T cells by GM-CSF Transduced 4T1. abstract # 55.6 FASEB/AAI, May 12-16, 2000

**APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples of appendices include journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.
Morphology and Flow Phenotype of 4T1

Figure 1: The parental 4T1 cell line was analyzed by FACS for expression of H-2K\textsuperscript{d}, CD80, CD86, CD40, and ICAM-1. A photomicrograph of a representative field of a flask containing the 4T1 cell line in log growth phase is shown above.
Morphology and Flow Phenotype of 4T1-7

Figure 2. The 4T1-7 subclone of the parental 4T1 cell line was analyzed by FACS for expression of H-2K$^d$, CD80, CD86, CD40, and ICAM-1. A photomicrograph of a representative field of a flask containing the 4T1-7 subclone in log growth phase is shown above.
Figure 3. The 4T1-9 subclone of the parental 4T1 cell line was analyzed by FACS for expression of H-2K\(^d\), CD80, CD86, CD40, and ICAM-1. A photomicrograph of a representative field of a flask containing the 4T1-9 subclone in log growth phase is shown above.
Figure 4. Cytokine release assay with effector T cells generated from lymph nodes draining either the 4T1-9 clone or the 4T1 clone secreting GM-CSF (E10-9). Effector T cells (2x10^6 / well in a 24 well plate) were cultured with either immobilized anti-CD3 (positive control), or stimulated with the parental 4T1 or the unrelated syngeneic breast cancer cell line, EMT6. Background cytokine release was determined by culturing the effector T cells alone (none).
Figure 5. **Unpublished data that should be protected.**

Effector T cells generated from 4T1-E10-9 TVDLN were either unseparated (E10-9), or separated into CD4+ or CD8+ subsets using miltenyi beads. Resulting populations were assayed for CD4 or CD8 expression by FACS and tumor-specific IFN-γ release by ELISA.
Table I  GM-CSF secreting 4T1 vaccine Improves Therapeutic Efficacy

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<th>Tumor Vaccine</th>
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<th>IL-2</th>
<th># of pulmonary metastases</th>
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<td>None</td>
<td>+</td>
<td>25, 250, 250, 250, 250</td>
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<tr>
<td>4T1-9</td>
<td>30 X 10^6 CD62L^Lo</td>
<td>+</td>
<td>181, 250, 250, 250, 250</td>
<td>236 (14)</td>
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<tr>
<td>E10-9</td>
<td>30 X 10^6 CD62L^Lo</td>
<td>+</td>
<td>3, 3, 7, 10, 12</td>
<td>7 (2)^*</td>
</tr>
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</table>

* p<0.05 between E10-9 group and 4T1-9 group or IL-2 control

IL-2 Control  30 X 10^6 4T1-9 CD62L^Lo TVDLN cells  30 X 10^6 E10-9 CD62L^Lo TVDLN cells
IFN-γ ELISA of STAT6−/− or WT TVDLN Effector cells

![Bar chart showing cytokine release assay results](image)

Figure 7. **Unpublished data that should be protected.** Cytokine release assay with effector T cells generated from TVDLN of either wild type (wt) or STAT-6 ko mice that were vaccinated with the GM-CSF secreting 4T1 clone (E10-9). Effector T cells (2x10^6 / well in a 24 well plate) were cultured with either immobilized anti-CD3 (positive control), or stimulated with the parental 4T1 or the unrelated syngeneic breast cancer cell line, EMT6. Background cytokine release was determined by culturing the effector T cells alone (none).
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Deputy Chief of Staff for Information Management