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14. ABSTRACT

During the last research period, we have made significant progress in the development of our mouse neu-reactive T cell lines. First, we have confirmed the key CD4+ neu peptides (p101 and p373) most effective at priming T cell responses. Of the peptide-specific T cell lines tested, only p101- and p373- T cells induced both peptide- and protein-specific responses. In preliminary studies involving adjuvants, GM-CSF was highly effective for use with our peptide vaccines. Second, we have thoroughly characterized the cytokine production by our ex vivo expanded T cells and observed high levels of secreted Th1 cytokines, primarily IFNγ and GM-CSF, but also, interestingly, Th2 cytokines, primarily IL-5 and IL-6. Furthermore, IL-17, a pro-inflammatory cytokine, was also found to be secreted by the cultured T cells during ex vivo expansion. Our studies have also demonstrated that the addition of IL-7 (among all the tested cytokines) to IL-2 in the T cell culture regimen is necessary for optimal ex vivo growth. Third, we have clearly demonstrated that our neu specific T cell lines were highly effective at inhibiting tumor growth, particularly MP (multiple peptide)-specific T cells. Three T cell infusions were more effective at inducing tumor regression than a single infusion. Most importantly, the antitumor inhibition was mediated by endogenous CD8+ T cells.

15. SUBJECT TERMS
Neu-transgenic mice, neu antigen, peptides, vaccines, CD4+ T cells, cytokines, adoptive T cell therapy.

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Introduction

The goal of this study is to evaluate mechanisms of generating $CD4^+$ neu-specific T cells *ex vivo* and to evaluate their anti-tumor efficacy *in vivo* in the neu-transgenic (neu-Tg) mouse model. Despite advances made in breast cancer research, relapse of micrometastatic breast disease remains to be a major hurdle. In animal models, adoptive T cell therapy has been shown to be an effective treatment for breast cancer. However, the potency of antitumor specific T cell cells can be limited by the number and type of T cells generated *ex vivo*, by endogenous T regulatory cells (Tregs) in the tumor microenvironment dampening the immune response, and the supoptimal ability of infused T cells to home to tumor and mediate tissue destruction. In addressing some of these challenges, the study has three specific aims: (1) to generate neu-specific CD4+ Th1 T cells in neu-Tg mice and determine whether infusion of these cells results in a long lasting anti-tumor response and epitope spreading; (2) to determine whether depletion of Tregs prior to infusion of tumor-specific CD4+ Th1 cells will enhance *in vivo* T cell proliferation and persistence thus augmenting anti-tumor therapeutic efficacy; and (3) to determine whether neu-specific T cells can home to micrometastatic tumor *in vivo* and elicit tumor cell apoptosis.

Body (Research Accomplishments)

Specific Aim 1: To generate neu specific CD4+ Th1 T cells in neu-Tg mice and determine whether infusion of these cells results in an anti-tumor response and epitope spreading.

A.1. To elicit a high magnitude neu specific Th1 response after active immunization in preparation for *ex vivo* expansion. Table 1 (Appendices) shows the neu peptides previously identified in our lab using peptide prediction algorithms. neu-Tg mice were immunized with each peptide three times at a 50 μg vaccine dose (with CFA/IFA as adjuvant) and then spleens were harvested. The magnitude of peptide and tumor specific IFN-γ producing T cells were assessed by enzyme-linked immunosorbent spot (ELISPOT) assay. Peptide-specific T cell responses were detectable following p101, p373, p45, p780, or p420 immunization (Fig. 1A); these peptides have historically stimulated responses in a rat model. While these immunizations induced peptide responses, only a few induced protein responses. The p101 and p373 vaccinations consistently generated T cell responses to neu protein (i.e. lysate from mouse mammary carcinoma (MMC) cells which highly express neu) (Fig. 1B).

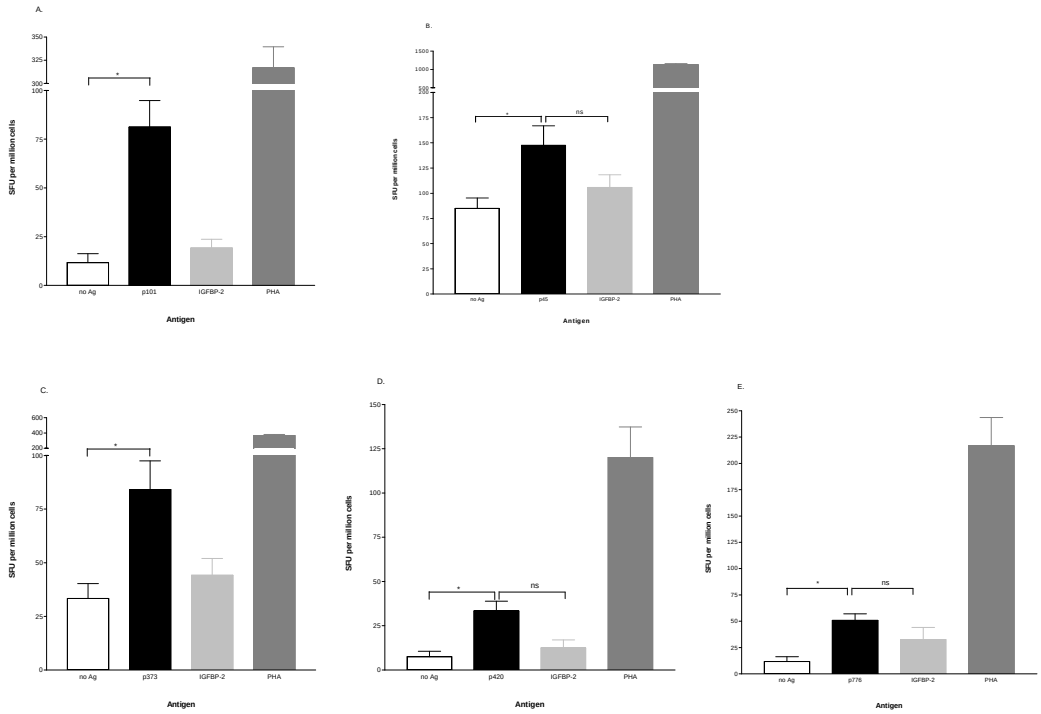

Figure 1A. IFN-γ ELISPOT of neu specific T cells. Shown are the spot forming units per million T cells for p101- (A), p45- (B), p373- (C), p420- (D) and p776-T cells (E) in response to the immunizing peptide.

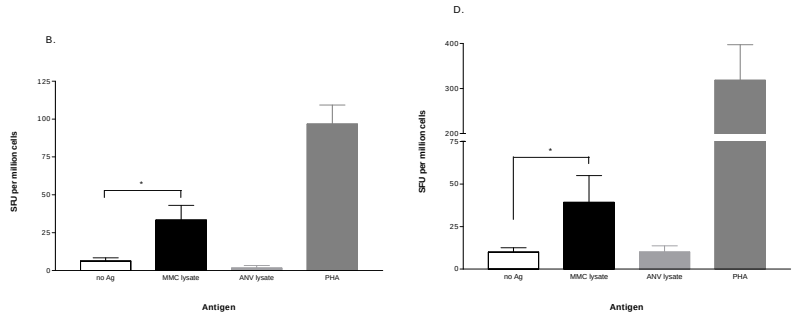

Figure 1B. IFN-γ ELISPOT of p101 and p373 neu specific T cells. Shown are the spot forming units for p101 T cells (A) or p373 T cells (B) in response to MMC protein.

Since the type of vaccine adjuvant used in peptide immunization can also influence the magnitude and phenotype of the T cell responses, we next examined various adjuvants (CFA, GM-CSF, IL-12 and imiquimod) for their ability to enhance the immunogenicity of the peptide vaccine. We evaluated the efficacy of each adjuvant to enhance p101 vaccination and protect from tumor development/growth. The doses used were 5 μg GM-CSF, 10 ng IL-12, and 5% imiquimod in a 3 day application with a single peptide, p101, at a 100 μg vaccine dose. In our preliminary experiment, at day 28 following tumor challenge, mice which received GM-CSF or IL-12 as the vaccine adjuvant had smaller tumors than those which received CFA (Fig. 2). IMQ treated mice had tumors of similar size as those which received CFA as a vaccine adjuvant. Current studies are underway to repeat these preliminary experiments *in vivo*. In addition, we are conducting *in vitro* ELISPOT assays to discern if,

indeed, there is any statistical difference between CFA (currently the standard adjuvant used in our laboratory) and the other adjuvants, particularly GM-CSF.

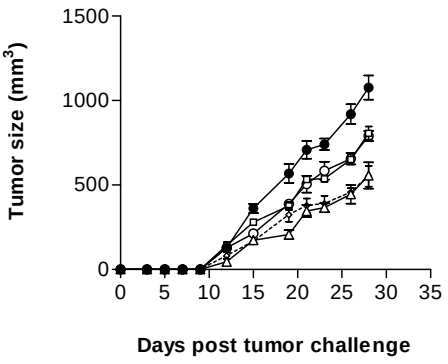

Figure 2. Comparison of vaccine adjuvants in neu-Tg mice. Mice (5 per group) were vaccinated with p101 peptide and adjuvant on days -42, -28, and -14, and challenged with live MMC cells s.c. on day 0. Adjuvants included CFA (open circles), imiquimod (open squares), GM-CSF (open triangles), or IL-12 (diamonds, dotted line). Control mice (n=4) received p101 peptide in PBS (closed circles). Tumor growth was followed after tumor challenge. Each data point represents the mean (±SEM).
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A.2. To modify the culture environment to augment the proliferation of Th1 CD4+ neu specific T cells. In the past, we had examined two representative Th1 cytokines (IFN-g and TNF-a) and two representative Th2 cytokines (IL-10 and IL-4) and found that the neu-specific T cell lines secreted high levels of these Th1 cytokines but low levels of Th2 cytokines. Within this past year, we conducted a more thorough characterization of cytokine secretion by the T cells lines. Using a multiplex cytokine assay (Luminex), we evaluated 5 Th1 and 5 Th2 cytokines in the supernatant of expanded p101- and p373-specific T cells (Fig. 3). While we found that Th1 cytokines, particularly GM-CSF and IFN-g, were highly secreted, we also detected high levels of some Th2 cytokines, such as IL-5 and IL-6. Therefore, we cannot with certainty conclude that our lines are Th1. We are currently confirming this characterization.

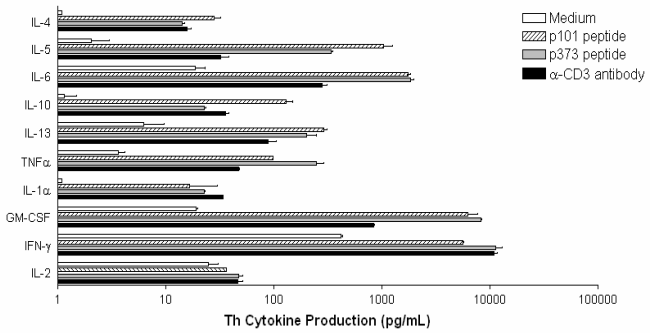

Figure 3. Multiplex cytokine analysis of expanded T cell lines. T cell lines were generated from spleens of p101 peptide-primed (dashed bar) or p373 peptide-primed (grey bar) mice. Supernatants of cultured T cells were collected 72 h after the second *in vitro* stimulation to evaluate for cytokine secretion using Luminex technology. Shown are the levels of Th1 (IL-2, IFN-g, GM-CSF, IL-1, TNF-a) and Th2 (IL-4, IL-5, IL-6, IL-10 and IL-13) detected in the culture supernatants. Control supernatants were collected from medium of unstimulated T cells in culture (white bar) or anti-CD3 stimulated T cells in culture (black bar).

Moreover, in preliminary studies, we detected IL-17 secretion by each of our peptide specific T cell lines, particularly by MP (multiple peptide)-specific T cells, which recognize p101, p373 and p45 (Fig. 4). IL-17 is a proinflammatory cytokine, produced by activated CD4+ T cells, that has been linked to antitumor effects [1]. Recently, the subset of T cells that secrete IL-17 has been defined as Th17 cells, which are distinct from Th1 and Th2 cells [2]. These cells are important in mediating autoimmunity and tissue inflammation, thus, it is possible that our expanded T cells can promote the generation of a Th17 response which may help augment antitumor inhibition (for *in vivo* tumor inhibition studies, see A.3).

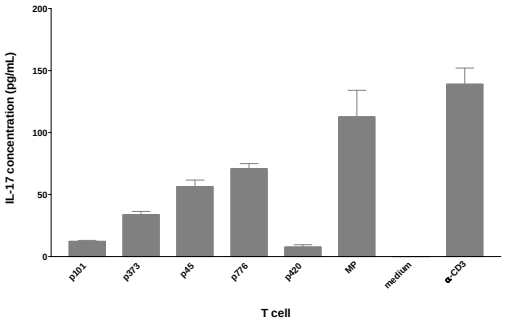

Figure 4. IL-17 is secreted by neu specific T cells. T cell lines were generated from spleens of peptide-primed mice. Supernatants of cultured T cells were collected 72 h after the second *in vitro* stimulation and evaluated for levels of IL-17. Control supernatants were collected from the medium of unstimulated T cells and anti-CD3 stimulated T cells in culture.

For this subaim, we also evaluated strategies of expanding T cells using different cytokines in the culture medium. Preliminary studies were done evaluating culture regimens that included: (1) IL-2, (2) IL-2+IL-7, and (3) IL-2+IL-7+anti-IL10. By multiplex cytokine assay on cultured T cell supernatants, we found that in the IL-2 and IL-2+IL-7 conditions, Th1 cytokines (GM-CSF and IFN-g) and Th2 cytokines (IL-5 and IL-6), were highly secreted; concentrations of these cytokines were 1000 pg/mL or greater (Fig. 5A, B). Concentrations of IL-5 and IL-6 decreased after the addition of anti-IL10 to the culture. The level of IL-10, a highly immunosuppressive cytokine, became undetectable after addition of anti-IL10 antibody (Fig. 5B).

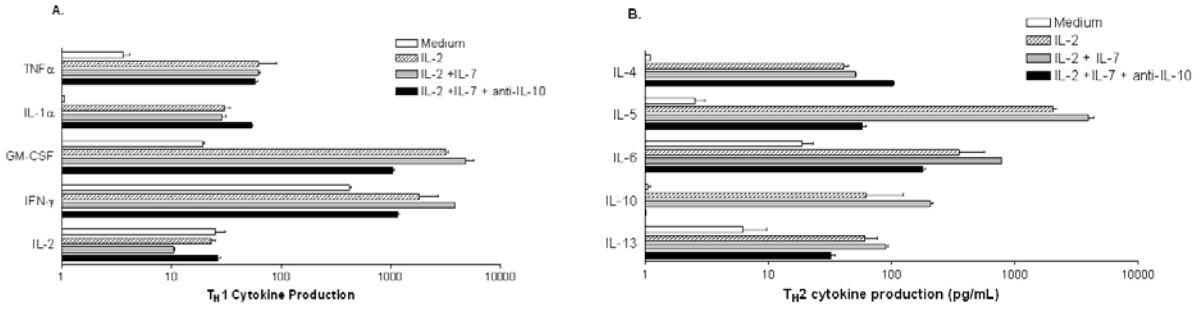

Figure 5. Multiplex cytokine analysis of expanded T cell lines cultured using various cytokines. T cell lines were generated from spleens of p101 peptide-primed or p373 peptide-primed mice, and cultured in: (1) IL-2 alone, (2) IL-2+IL-7, or (3) IL-2+IL-7+anti-IL10. Supernatants of cultured T cells were collected 72 h after the second *in vitro* stimulation to evaluate for cytokine secretion using Luminex technology. Shown are the levels of Th1 (A) and Th2 (B) cytokines. Control supernatants were collected from medium of unstimulated T cells in culture.

This past month, we have begun a thorough comparative analysis of T cell culture regimens. The T cells were cultured with the following cytokines: (1) IL-2, (2) IL-2+IL-7, (3) IL-2+IL-12, (4) IL-2+IL-15, (5) IL-2+IL-18, (6) IL-2+IL-21, (7) IL-2+anti-IL-10, and (8) IL-2+anti-TGF-b (Fig. 6). Thus far, it appears that the addition of IL-7 in the culture medium stimulates maximal T cell growth (Fig. 6).

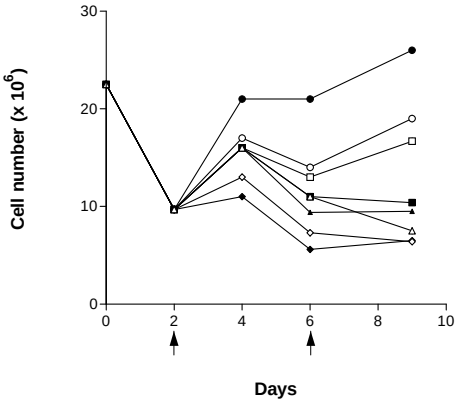

Figure 6. ***Ex vivo*** **growth of T cells cultured using various cytokines.** p101-T cells were harvested from mice, and equally separated into flasks, at a concentration of $2x10^6$ cell/mL and stimulated with peptide. The starting cell number is noted on day 0. On day 2, appropriate cytokines were added to the culture medium, and every 2-3 days thereafter. On day 6, the T cells received an *in vitro* stimulation. T cell numbers were collected periodically.. The cytokine conditions included: IL-2 (open triangle), IL-2+IL-7 (closed circle), IL-2+IL-12 (closed square), IL-2+IL-15 (open circle), IL-2+IL-18 (open diamond), IL-2+IL-21 (closed diamond), IL-2+anti-IL-10 (closed triangle), and IL-2+anti-TGF-b (open square).

A.3. To determine whether infusion of neu specific Th1 T cells results in an anti-tumor response and epitope spreading. From A.1, we determined that p101 and p373 T cells should go forward with expansion and be tested *in vivo* for their ability to induce tumor regression. Since it might also be useful to compare them to T cells which responded to peptide but not to protein, we also generated T cell lines for the other peptides (p45, p776, p420), as well a T cell line recognizing multiple peptides (p101, p373, and p45). As seen in Fig. 6, tumor growth was inhibited following infusion of 10 x 10^6 of p101 or p373 neu specific T cells (about 60% inhibition of tumor growth compared to control T cells) (Fig. 7). Tumor inhibition with p45, p776, p420 T cell lines also showed about 60% inhibition (data not shown). Therefore, while these peptide-specific T cell lines, unlike p101 and p373 T cells, did not exhibit protein specificity by ELISPOT, they appeared equally effective *in vivo* at mediating tumor regression. We are currently assessing the avidity of our T cell lines (by ELISPOT) to determine if this may account for poor detection of *in vitro* peptide responses. We also found that 3 T cell infusions were more effective than one at inducing tumor regression. As well, we found that multiple peptide (MP)-specific T cells were more effective than single peptide-specific T cells in inhibiting tumor growth (Fig. 8). At day 21, tumor inhibition by MP T cells compared to control T cells was 81% (p=0.0423) (Fig. 8).

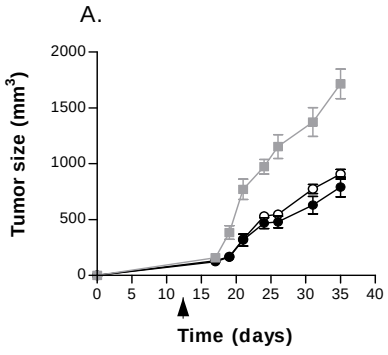

Figure 7. p101 and p373 T cells inhibit tumor growth. Mice (5 per group) were given live MMC cells s.c. on day 0. On day 12 (arrow) following tumor implantation, mice were injected i.v. with 10 x 10^6 p101-T cells (closed circles), p373-T cells (open circles), or naïve syngeneic T cells (closed squares). Tumor growth was followed. Each data point represents the mean (±SEM).

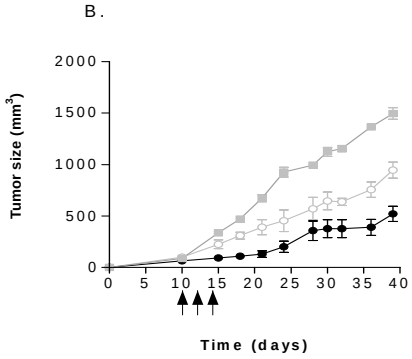

Figure 8. Multiple peptide-recognizing T cells inhibit tumor growth. Mice (3 per group) were given live MMC cells s.c. on day 0. On days 10, 12, and 14 (arrows), mice were injected i.v. with 10 x 10^6 p373 T cells (open circles), multiple peptide (p101, p373 and p45) specific T cells (closed circles) and control T cells (T cells pulsed briefly with p373 peptide; closed squares). Tumor growth was followed. Each data point represents the mean (±SEM).

To investigate the effector arm responsible for mediating the antitumor response, mice were depleted of CD8+ T cells, CD4+ T cells and B cells prior to T cell infusion. Anti-CD8 (150 µg in 150 µl), anti-CD4 (250 µg in 250 µl) or anti-B cell (160 µg in 160 µl) antibodies were injected into the appropriate groups of tumor-bearing mice, on the same day that p373-T cell infusion was initiated (day 12). The mice received three consecutive injections of appropriate antibody in the first week, followed thereafter with two injections weekly until the end of the study. Only those which lacked endogenous CD8+ T cells showed an abrogation of tumor rejection, thus, indicating that tumor rejection is mediated by CD8+ T cells (Fig. 9A). Endogenous CD4+ T cells and B cells do not appear to be as important since their depletion had no effect. We do recognize that there is a possibility the antibodies may also deplete and/or inhibit the infused T cells. Therefore, we administered the anti-CD8 antibody only prior to T cell infusion (days 5, 6, 7) and saw similar trends as before (Fig. 9B). Assessment of epitope spreading will be done in Year 2 (see Future Directions).

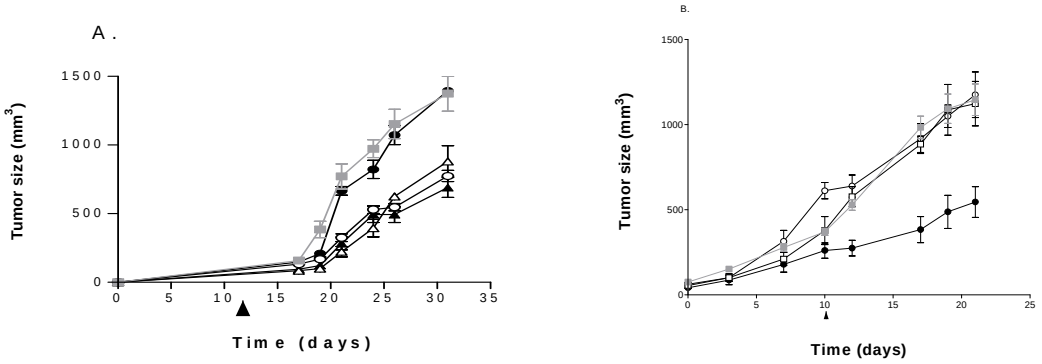

Figure 9. Depletion of endogenous CD8 T cells abrogates therapy of infused neu specific T cells. Mice (n=4) were given live MMC cells s.c. on day 0. Following tumor implantation, T cells were infused on the same day (A) or after administration of depleting antibodies (B). In (A), 12 days following tumor implantation, mice were infused (arrow) with 10 x 10^6 p373-T cells in the absence (open circles) or presence of CD8-depleting antibodies (closed circles), CD4-depleting antibodies (open triangles), or B-cell depleting antibodies (closed triangles). Control mice were infused with T cells briefly pulsed with p373 peptide (closed squares). In (B), p101- T cells were infused on day 10 (arrow) either alone (closed circles) or with CD8-depleting antibodies (open circles). Control mice received naïve T cells, infused alone (closed squares) or with CD8-depleting antibodies (open squares). Antibody was administered on days 5, 6, and 7.

Specific Aim 2: To determine whether depletion of Tregs prior to infusion of tumor specific CD4+ Th1 cells will enhance *in vivo* T cell proliferation and persistence, thus augmenting anti-tumor therapeutic efficacy.

These experiments will begin in months 24-36, as outlined in the original statement of work (SOW).

Specific Aim 3: To determine whether neu-specific T cells can home to micro-metastatic tumor *in vivo* and elicit tumor cell apoptosis.

The mouse PET scanner is still in development. Therefore, this past year, more efforts have been placed on specific aim/task 1, particularly assessment of the antitumor efficacy of the

neu T cell lines *in vivo* (see S.A. 1). Nonetheless, in preparation for the *in vivo* evaluation of apoptosis induced by infused T cells, we tested an *in vitro* assay to assess/quantify the level of apoptotic death. Using a FACS-based assay to quantitate Annexin-V positive cells, we analyzed tumors of mice after treatment with a standard chemotherapy, e.g. doxorubicin (Fig. 10). After harvest, tumor cells were co-stained with Annexin-V FITC and propidium iodide (PI). Viable cells are Annexin$^-$/PI$^-$, necrotic cells are Annexin$^+$/PI$^+$ and apoptotic cells are Annexin$^+$/PI$^-$. The percent of apoptotic cells detected after doxorubicin treatment was 2 times greater than PBS treatment (41% vs. 21%) (Fig. 10). Thus, an assessment of apoptosis, such as this one, can be used as a additional means to confirm *in vivo* detection of apoptosis.

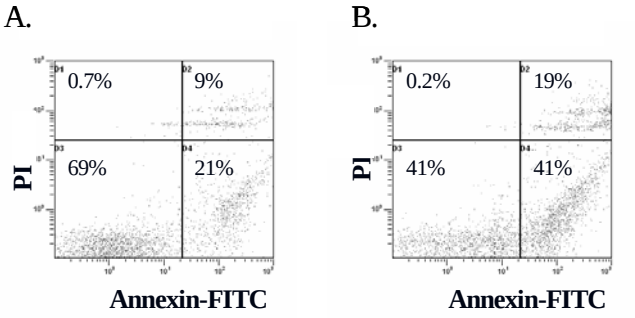

Figure 10. Detection of apoptostic cells by Annexin-V FACS. Tumors from mice treated with PBS (A) or doxorubicin (B) were harvested and labeled with Annexin V-FITC and Propidium Iodide (PI) antibodies.

Key Research Accomplishments

- The key CD4+ neu peptides most effective at priming T cell responses are p101 and p373; of the peptide-specific T cell lines tested, only p101- and p373- T cells induced both peptide- *and* protein-specific responses
- GM-CSF is highly effective for use with our peptide vaccines
- Thorough characterization of the cytokine production by our *ex vivo* expanded T cells reveal high levels of secreted Th1 cytokines, primarily IFNγ and GM-CSF, but also Th2 cytokines, primarily IL-5 and IL-6
- IL-17, a pro-inflammatory cytokine, is also secreted by the cultured T cells during *ex vivo* expansion
- Addition of IL-7 (among all the tested cytokines) to IL-2 in the T cell culture regimen is necessary for optimal *ex vivo* growth
- neu specific T cell lines, particularly MP (multiple peptide)-specific T cells, are highly effective at inhibiting tumor growth,
- Three T cell infusions are more effective at inducing tumor regression than a single infusion
- Antitumor inhibition following infusion of neu specific T cells is mediated by endogenous CD8+ T cells.

Reportable Outcomes

Publications

Phan, V., Saddoughi, S., Gad, E., Dang, Y., Park, K-H, and Disis, M.L. Identification of Rat *Neu*-specific T-helper Epitopes that Elicit CD4+ Th1 Cells Which Mediate an Anti-tumor Response in *Neu*-transgenic Mice. Poster presentation at *AACR Special Conference on Tumor Immunology: An Integrated Perspective*, November 29 - December 2, 2006, Miami, FL.

Phan, V. and Disis, M.L. Tumor stromal barriers to the success of adoptive T cell therapy. Commentary in *Cancer Immunol Immunother*, July 2007.

Phan, V., Gad, E., Saddoughi, S., Waisman, Z., DeLong, J., and Disis, M.L. Targeting HER2-specific CD4+ Th1 cells to tumors to induce CD8+ T cell-mediated tumor rejection. Poster presentation at International Society for Biological Therapy of Cancer (iSBTc) meeting, to be held November 2-4, 2007, Boston, MA.

Conclusions and Future Directions

During the last funding year, we have made significant progress in the way we generate our mouse neu-reactive T cell lines. First, we have confirmed the key CD4+ neu peptides (p101 and p373) most effective at priming T cell responses. In preliminary studies involving vaccine adjuvants, there is strong suggestion for the use of GM-CSF with our peptide vaccines. Second, we have thoroughly characterized the cytokine production by our *ex vivo* expanded T cells and, interestingly, observed high levels of secreted Th1 cytokines, primarily IFNg and GM-CSF, but also Th2 cytokines, primarily IL-5 and IL-6. Our studies have also demonstrated that the addition of IL-7 to IL-2 in the T cell culture regimen is necessary for optimal *ex vivo* growth. Third, we have clearly demonstrated that our neu specific T cell lines are highly effective at inhibiting tumor growth and, importantly, that the mechanism is mediated by endogenous CD8+ T cells.

Future directions are as follows:
Specific Aim 1: We will continue to identify the optimal mouse T cell culture regimens by characterization of their cytokine profile, growth properties, *and* antitumor properties *in vivo*. Epitope spreading will be assessed by the following means: 1) FACS evaluation for p420 (CD8+ neu peptide)-recognizing T cells isolated from tumors of CD4+ T cell-treated mice using a p420 *neu* tetramer; and 2) serological analysis of cDNA expression library (SEREX) of mouse serum pre- and post- T cell therapy to assess for antibody responses to other antigens besides neu.
Specific Aim 2: In the upcoming year, we will evaluate the use of immunomodulatory and/or T regulatory-depleting agents, such as cytoxan or ONTAK, to augment adoptive T cell therapy. We recognize that infusion of the neu-specific lines did not induce complete tumor inhibition. Lymphodepletion with cytoxan prior to T cell infusion may create a homeostatic space to enable enhanced proliferation of infused T cells, and may augment antitumor efficacy of infused T cells. Thus, we will evaluate infusion of antigen-specific T cells in mice pretreated with cytoxan. We will also evaluate infusion of antigen-specific T cells following pretreatment with ONTAK and anti-CD25 antibody. In some studies, T cells will be labeled with CFSE prior to infusion to: 1) track homing of infused T cells to tumor, and 2) assess proliferation of infused T cells *in vivo*. After infusion, the tumors of treated

mice will be harvested and stained for CD4-PE and CD8-PECy7 antibodies to evaluate the percentage of CFSE+/CD4+ T cells and CFSE+/CD8+ T cells.

Specific Aim 3: Over the next couple years, studies will focus on specific aim 3 involving a multidisciplinary collaboration with nuclear medicine scientists and clinicians on the use radiotracers and PET modality to image effector T cell functions *in vivo*. We will test the biodistribution of the Annexin V radiotracer using 'cut and count' assays. First, we plan to inject neu-tg mice with doxorubicin (before testing our neu specific T cells) to optimize detection of the radiotracer. In the past, our lab has conducted radiotracer uptake studies examining tumor glucose metabolism and proliferation. In the proposed study, our goal is to evaluate tumor apoptosis as a biological response (in addition to metabolism and proliferation) that is likely to be altered within tumor cells after adoptive T cell therapy.

References:

1. Benchetrit, F., et al., *Interleukin-17 inhibits tumor growth by means of a T-cell-dependent mechanism.* Blood, 2002. **99** (6): p. 2114-21.
2. Wilson, N.J., et al., *Development, cytokine profile and function of human interleukin 17-producing helper T cells.* Nat Immunol, 2007. **8** (9): p. 950-7.

Appendices:

Table 1. Sequences of rat *neu* peptides.

Rat neu peptide	*Amino acid*	*Sequence*
p101	101-117	RLRIVRGTQLFEDKYAL
p373	373-340	KIFGSLAFLPESFDGDPS
p45	45-59	HLDMLRHLYQGCQVV
p780	780-794	GVGSPYVSRLLGICL
p420	420-429	PDSLRDLSVF
p61	61-74	NLELTYLPANASLS
p83	83-96	QGYMLIAHNRVKHV
p668	668-685	LIIVVVIGILIKRRRQKI