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13. ABSTRACT *(Maximum 200 Words)*

The identification and characterization of tumor antigens has facilitated the development of immune-based cancer prophylaxis and therapy. Cancer vaccines, like viral vaccines, may be effective in cancer prevention. Adoptive T cell therapy, in contrast, may be more efficacious for the eradication of existing malignancies. I am examining the feasibility of antigen-specific adoptive T cell therapy for the treatment of established cancer in the HER-2/neu model. Transgenic mice overexpressing rat neu in mammary tissue develop malignancy, histologically similar to human HER-2/neu^{+} breast cancer. These mice can be effectively immunized against a challenge with neu^{+} tumor cells. Adoptive transfer of neu-specific T cells into tumor-bearing mice eradicates malignancy. Effective T cell therapy relies on optimization of the *ex vivo* expansion of antigen-specific T cells. Two important elements of *ex vivo* antigen-specific T cell growth that have been identified are (1) the pre-existing levels of antigen-specific T cells, and, (2) the cytokine milieu used during *ex vivo* expansion of the T cells. Phase I clinical trials of HER-2/neu-based peptide vaccination in human cancer patients have demonstrated that increased levels of HER-2/neu-specific T cells can be elicited after active immunization. Initiating cultures with greater numbers of antigen-specific T cell lines and clones facilitates expansion. In addition, cytokines, such as IL-12, when added during *ex vivo* culturing along with IL-2 can selectively expand antigen-specific T cells. IL-12 also enhances antigen-specific functional measurements such as IFN-γ and TNF-α release. Refinements in *ex vivo* expansion techniques may greatly improve the feasibility of tumor-antigen T cell-based therapy for the treatment of advanced stage HER-2/neu-overexpressing breast malignancy.

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Introduction

The administration of large numbers of tumor-specific T cells has been shown to be an effective method of eradicating tumors in animal models and in treating established infectious disease in immunocompromised patients [1-3]. Augmenting a tumor-specific immune response offers a treatment that potentially has, (1) specificity, (2) the ability to eradicate all circulating tumor cells, and, (3) minimal toxicity. However, early attempts to apply this approach to human cancers have met with limited success. Initially, based on the assumption that the greatest numbers of tumor-specific lymphocytes would be found infiltrating tumors themselves or in regional tumor-draining lymph nodes, most adoptive immunotherapy strategies entailed non-specific expansion of TIL (tumor infiltrating lymphocytes). While TIL could be expanded, infusions did not result in many clinical responses. Recent advances in molecular immunology providing the scientific technology needed to define tumor-specific antigens and an understanding of how T cells recognize antigen have made it possible to consider strategies using antigen-specific adoptive T cell immunotherapy [4]. Indeed, defining what proteins in melanoma are immunogenic has allowed for analysis of TIL for antigen-specificity. For example, Kawakami reported that it was the adoptive transfer of TIL that were reactive to the melanoma antigen gp100 that was associated with a clinical tumor response [5]. For the last several years our group has been studying the HER-2/neu (HER2) oncoprotein as a tumor antigen in several solid tumors such as breast, ovarian, and non-small cell lung cancer. Our group has developed vaccine strategies targeting HER2 and have conducted Phase I clinical trials of peptide-based vaccines which have been effective in significantly increasing the HER2-specific T cell precursors, both cytotoxic (CTL) and T helper (Th), in immunized patients. Our central hypothesis is that while vaccines may be useful in preventing cancer relapse, eradicating established tumors in cancer patients will require the generation of high levels of tumor-specific immunity, levels which can not be achieved by vaccination, but rather by infusion of competent T cells, i.e. adoptive T cell therapy (7).

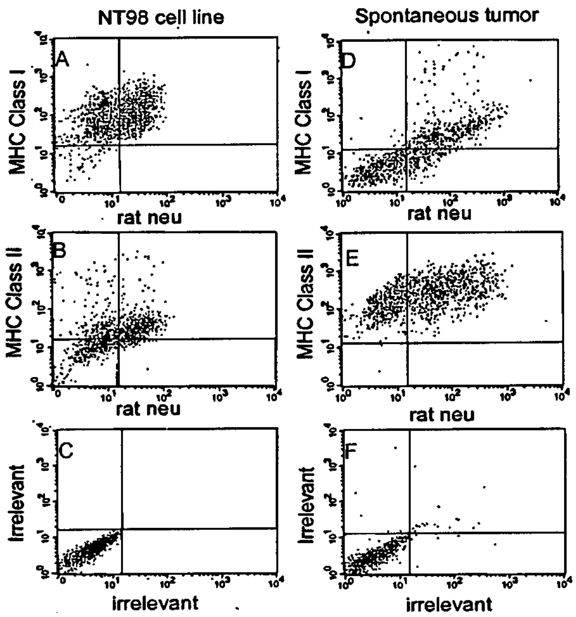


Figure 1: NTT9-98 cell line stably expresses neu protein as well as MHC claass I and MHC class II. Spontaneous tumor from a neu-Tg mouse was minced and tumor cells purified by gradient centrifugation. Tumor cells were seeded at 5 x 10^6 cells in T25 flasks in 20% FCS in RPMI (GIBCO). After 30 days in culture a cell line was established which overexpresses rat neu. NTT-98 was expanded and aliquots frozen at the 3rd and 5th passages. Flow cytometry was performed at the 5th passage (panels A-C), staining for neu, MHC class I, and MHC class II. Results are compared with tumor cells purified from an early (1-2 month) primary spontaneous tumor (panels D-F). Cells were stained with (C, F) irrelevant antibody, (A, D) c-neu-FITC and anti-MHC class I-PE or (B, E) anti-MHC class II-PE.

This progress report details the pre-clinical studies accomplished pertaining to the specific aims of

A. <u>To determine the immune effector arm most effective in the eradication of rat neu overexpressing tumors in neu-tg mice using adoptive immunotherapy of rat neu-specific $CD8^+$ and $CD4^+$ T cells</u>, and,

B. <u>To determine the optimal *ex vivo* conditions for the expansion of HER2-specific $CD4^+$ and $CD8^+$ T cell lines from patients previously immunized with a HER2 peptide-based vaccine</u>.

Body

I. Specific Aim A: To determine the immune effector arm most effective in the eradication of rat neu-overexpressing tumors in neu-tg mice using adoptive immunotherapy.

A. A neu+ tumor cell line from neu-tg mice maintains MHC class I and II expression in vivo. Neu-tg mice are engineered to express non-transforming (nonmutated) rat neu on an MMTV promoter [6]. Although 80% of the mice spontaneously develop breast tumors, the time to tumor development varies and less predictable than desired. We have developed a tumor transplant system using a cell line derived from a naturally occurring neu-mediated tumor in the neu-tg mice. A tumor cell line, NTT9-98, derived from a spontaneous neu-tg tumor was established in culture. We have characterized this cell line and use it to generate synchronized tumors in mice. *Figure 1* shows flow cytometric analysis of the cultured tumor cell line after 5 passages compared to an early stage (i.e. 1-2 month old) primary tumor. Greater than 98% of the cell line expressed MHC class I and greater than 80% of the cell expressed MHC class II. The primary tumor cells also expressed MHC class I and class II.

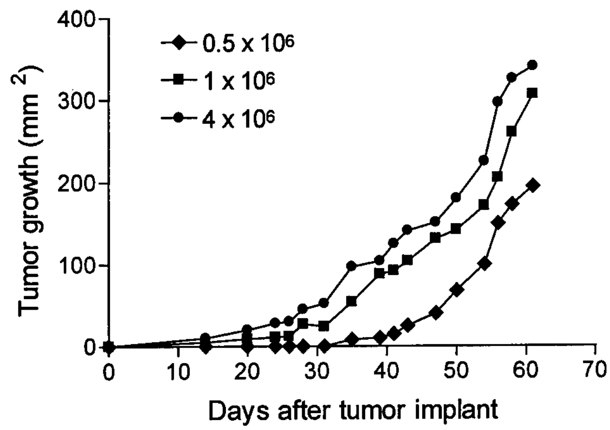


Figure 2. Mammary carcinomas derived from neu transgenic mice, cultured *in vitro* for >60 days, are tumorigenic in non-tumor bearing neu transgenic mice. 0.5x10^6, 1.0x10^6, or 4.0x10^6 NT98 cells were implanted subcutaneously in non-tumor bearing *neu*-tg mice. All mice demonstrated tumor growth. The kinetics of growth was dependent on dose of tumor given. Data for each experimental group represents the mean value of 5 mice. No tumors developed in parental animals implanted with the varying doses of NT98 (all groups shown together at the X axis).

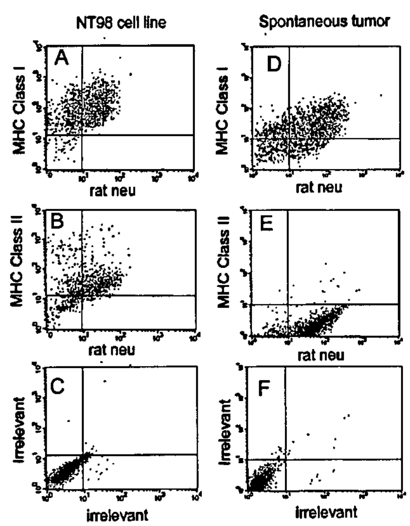


Figure 3: NTT9-98 cell line stably expresses neu protein as well as MHC claass I and MHC class II after >60 days *in vivo* growth. NTT9-98 cells were grown in vivo for >60 days. The tumor resulting from the NTT9-98 injection (panels A-C) and a late stage (>60 days) spontaneous tumor (panels D-F) were removed and compared for expression of MHC class I and class II as described in Figure 1. Cells were stained with (C, F) irrelevant antibody, (A, D) c-neu-FITC and anti-MHC class I-PE or (B, E) anti-MHC class II-PE.

B. NTT9-98 tumor cell line dose-dependently generates tumors in neu-Tg mice. My grant proposal outlines experiments designed to effector arm mediating tumor killing in both an established disease model and a minimal disease model. In order to determine if the NTT9-98 cell is suitable for both minimal and established, the tumorigenesis of the tumor cell line was tested at various concentrations. Figure 2 demonstrates that tumors were generated at all doses used. The higher dose of tumor (4 x 10^6) yielded tumors first appearing at about 18 days and the lower dose yielded tumors appearing at approximately 35 days.

C. NTT9-98 tumor cell line expresses MHC class I and class II after >60 days in vivo. Older (>60 days) spontaneous tumors in neu-Tg mice demonstrate variable expression of MHC class I and class I and would therefore be problematic when assessing the role of the T cell response. MHC class I and class II expression were tested

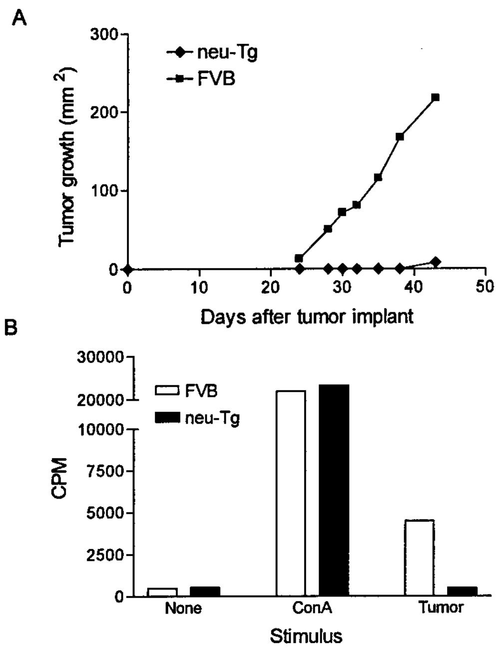


Figure 4. NTT9-98 tumor cell line is tumorigenic but not immunogenic in non-tumor bearing neu-tg mice but are immunogenic and not tumorigenic in parental FVB mice. 4.0×10^6 NT98 cells were implanted subcutaneously in 5 non-tumor bearing *neu*-tg mice and 5 FVB mice. All neu-tg mice demonstrated tumor growth (panel A). None of the FVB parentals developed tumor. Splenocytes derived from each mouse were tested in proliferation assays against NTT9-98 cells following tumor growth (panel B). Data for each experimental group represents the mean value of 5 mice.

on NTT9-98 tumor cells following >60 days growth *in vivo*. Figure3 demonstrates that NTT9-98 tumor cells maintain MHC class I and class II expression. In contrast, primary tumor cells derived from an older spontaneous tumor lost MHC class II expression. In addition, approximately 25% of spontaneous tumor lose MHC class I expression (data not shown). Approximately 90% of spontaneous tumors lose MHC class II expression with advanced age.

D. NTT9-98 tumor cells elicit an immune response in parental FVB mice but not neu-tg mice. It was predicted that NTT9-98 cells should grow unimpeded in neu-tg mice but not the parental strain FVB due to expression of the transgene. This was tested by injecting either neu-tg or FVB parentals with NTT9-98 tumor cells. As demonstrated in Figure 4, tumors were unable to grow in FVB parentals (panel A). The ability to reject tumor is likely due to the generation of an immune response to the tumor cells which is demonstrated in panel B.

E. neu-tg mice can be immunized with peptide-based vaccines. Previous studies in rats have shown that tolerance to rat neu can be circumvented with a peptide-based vaccine [7]. We immunized neu-tg mice with a vaccine composed of peptides derived from the ICD of rat neu. Similarly in neu-tg mice where rat neu is a self-protein, immunization with the peptide-based vaccine resulted in the generation of rat neu-specific antibodies (Figure 5A) and T cell immunity (Figure 5B).

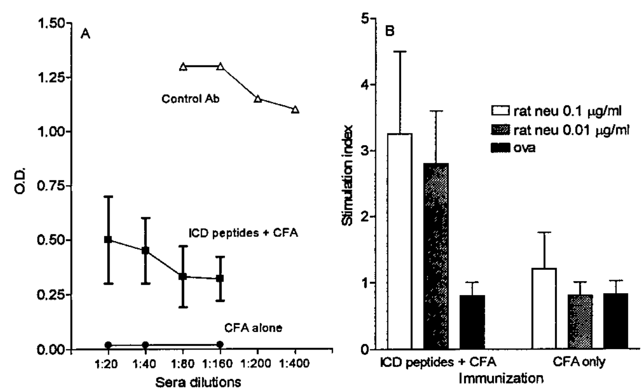


Figure 5. Neu-tg mice can be immunized to neu with a peptide based vaccine. Neu-tg mice were immunized with a neu ICD peptide based vaccine at 14 day intervals for 2 inoculations. Data described here represents neu specific antibody and T cell immunity generated by vaccination. Panel A depicts the neu protein-specific antibody response. Data is expressed as the mean and standard deviation of 5 mice/group. A MAb specific for neu is used as a positive control. Panel B represents T cell response to neu protein at 2 concentrations as well as an irrelevant antigen, ova albumin, after peptide immunization. T cell were derived from mouse spleen. Data is expressed as the mean and standard deviation of 5 mice/group.

F. neu-tg mice can be immunized with the human ICD protein. Human neu is nearly 90% identical to rat neu. The human ICD protein is available and is currently being used to generate HER-2/neu immunity in neu-tg mice. Neu-tg mice received 2 immunizations with of 100 µg/immunization/mouse 14 days apart. The immunizations were given subcutaneously. Fourteen days after the final immunization, splenocytes were harvested and immunity was assessed using a proliferation assay. As demonstrated in figure 6, neu-tg mice could be immunized with a protein-based vaccine. I am currently assessing whether neu-specific antibodies were elicited following immunization. Sera was obtained from each mouse and cryopreserved for antibody ELISA.

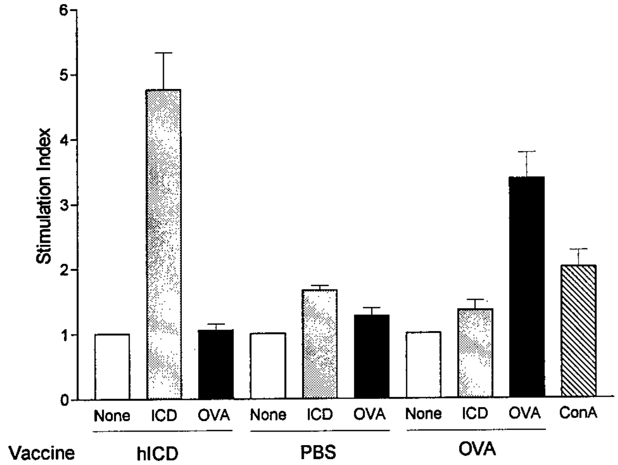


Figure 6. __**Neu-tg mice can be immunized to neu with a neu ICD protein-based vaccine.**__ Neu-tg mice were immunized with a human neu ICD protein-based vaccine, ovalbumin (OVA) protein-based vaccine, or PBS alone at 14 day intervals for 2 immunizations in CFA. Data described here represents neu-T cell or OVA T cell immunity generated by vaccination. T cells were derived from mouse spleen. Data is expressed as the mean and standard deviation of 5 mice/group. Concanavalin A (ConA) is used as a positive control for the assay. Mice from all three immunization groups were test for proliferation responses to PBS alone (white bar), 1 μg/ml human ICD protein (grey bar), or 1 μg/ml ovalbumin (black bar). Results are shown as the mean proliferation response of 12 wells for each stimulus (±sem). Similar results were observed in an independent experiment.

__G. Development of neu DNA-based vaccines.__ I have also began development of DNA-based neu vaccines encoding the full length rat neu, the ECD, and the ICD of rat neu (Figure 7). The vaccine were constructed in th vector, pNGVL3, produced by the National Gene Vector Laboratories (Indianapolis University, Indianapolis, Indiana). The vector uses a CMV promoter with expression enhanced by an intron, Intron A, which lies upstream of the gene insertion multiple cloning site. Since this vector is approved for use, the findings would be directly translatable to human clinical trials. The neu DNA was inserted directionally into the multiple cloning site using the restriction enzymes, *Not* I and *Sal* I, for the 3' and 5' termini, respectively. Following construction, all of the plasmids were tested The cell surface expression of with the full-length construct or the construct containing rat neu ECD were tested *in vitro* in chinese hamster ovary (CHO) cells to verify expression of the constructs. Figure 8 demonstrates that both the full-length and ECD constructs express rat neu and rat ECD, respectively. Forty-eight hours after transfection 30% of pNGVL3-Flrneu-transfected CHO cells expressed full-length rneu and 27% of pNGVL3-rneu-ECD-transfected CHO cells expressed the ECD. These results demonstrate that these constructs have the potential to express the neu inserts following immunization.

H. Vaccines, can eradicate established neu-expressing tumors in rodent models. Studies I have performed in a transgenic murine model of breast cancer demonstrate that vaccines are effective in protection and that T cell infusion is necessary for the treatment of established disease (Appendix A).

Mice immunized twice with an ICD peptid-based vaccine and then implanted with rat neu-expressing breast cancers rejected the tumors (*Figure 2A of Appendix A*). In these animals, the vaccine was protective only if mice had immunity at the time they were given the tumor challenge. However, if animals began the immunization schedule at the same time as the tumor was inoculated (i.e.,immunizing with a substantial tumor burden) there was no effect of peptide immunization in slowing tumor growth as compared to adjuvant alone (*Figure 2B of Appendix A*).

Development of these vaccination strategies paves the way for utilizing them to generate the *in vivo* immunity required to evaluate adoptive T cell therapy in this mouse model. Experiments are currently in progress to evaluate

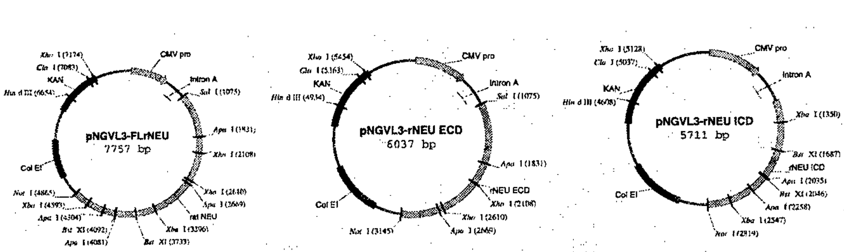


Figure 7. DNA vaccines encoding full-length rat neu, rat neu ECD, or rat neu ICD were constructed in a vaccine expression plasmid. The 3 plasmids constructed for neu DNA-based vaccination.

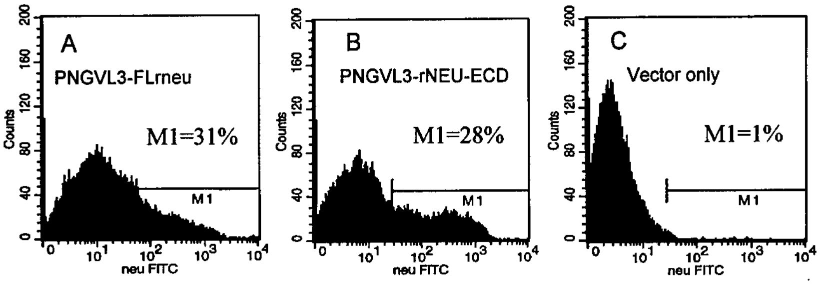


Figure 8. DNA vaccines encoding full-length rat neu, rat neu ECD, or rat neu ICD were constructed in a vaccine expression plasmid express the inserted genes in CHO cells. CHO cells were transiently transfected with pNGVL3-Flrneu (full-length nee, panel A), pNGVL3-rneu-ECD (extracellular domain, panel B), or pNGVL3 (vector control, panel C). neu expression was assessed by FACS analysis 48 hours following vaccination using FITC-conjugated anti-rat neu antibody.

specifically the immune effector arms critical for eradicating tumor following adoptive immunotherapy as well as determining the minimum number of effector T cells needed for therapy.

<u>I. Neu-specific T cells, derived from neu-tg mice, can be expanded *in vitro* and adoptive transfer of neu-specific T cells can inhibit the growth of neu overexpressing tumor *in vivo.*</u> Mice implanted with neu-tg tumors were treated with an infusion of rat neu-specific T cells generated in syngeneic animals that had been vaccinated with the ICD peptide vaccine. Briefly, immune spleens were removed from the animal and re-suspended at a cell number of 3-5x10^7 in RPMI/10%FCS. ICD peptides were added to this bulk culture at a concentration of 1.0 μM. Exogenous IL-2 was added (10 U/ml) on days 3, 5, and 9 of the culture cycle. The expanded T cell lines were used for infusion after 10 days in culture. Infusion of immune spleen taken from immunized syngeneic animals and directly transferred into tumor bearing mice without *in vitro* expansion also slowed tumor growth (Figure 3 of Appendix A). When the T cell population was enriched for neu-specific cells by an IVS with neu peptides, however, the therapeutic effect was more pronounced. In ongoing studies, I am currently identifying the most effective peptide-specific T cells generated as a result of peptide vaccination. I have immunized mice with a peptide-based vaccine consisting of two neu helper peptides, p781 and p1171. Following immunization, splenocytes were used to generate p781 or p1171-specific T cell lines which were subsequently re-infused into neu-tg mice with established tumors (i.e. 5 x 10^6 tumor cell injection)(Figure 9). Control mice were infused with either non-immune splenocytes or HBSS. As demonstrated in Figure 9, p1171-specific T cell lines, but not p781-specific T cell lines, were able to significantly inhibit growth of tumors. This is represents a very important step in identifying the T cell subsets that are more effective at tumor eradication in this model. Future studies will be directed at characterizing the reactive T cells (i.e. CD4 or CD8 T cells) as well as the numbers of cells needed for eradication.

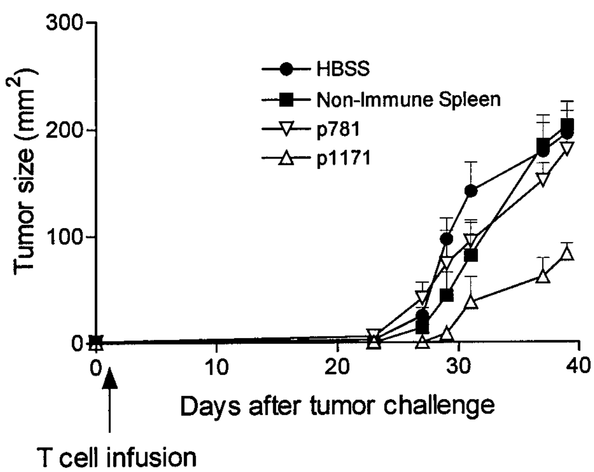


Figure 9: Adoptive transfer of neu peptide-specific T cells inhibits growth of neu-overexpressing tumors in vivo. Mice were immunized with neu ICD peptides p781 or p1171. Splenocytes were harvested and peptide-specific T cell lines were expanded ex vivo. The cell lines were re-infused in tumor-bearing mice. Tumor cells were implanted 1 day prior to T cell infusion at a dose of 5 x 10^6 tumor cells/mouse. Control mice received vehicle (HBSS) or non-immune splenocytes. Tumor growth was monitored for 40 days after which the experiment was terminated. Tumors were measured in 2 dimensions. Data is presented as the mean (±sem) of tumor dimensions from 5 mice/group.

<u>J. Murine, tumor-specific T cells can be identified in parental FVB mice and quantified by flow cytometry of trapped intracellular cytokines.</u> FVB mice, the parent strain of the neu-tg mice, were injected with 4 x 10^6 tumor cells. After 40 days, spleens were harvested and incubated in either media alone or with irradiated tumor for 5 days. On day 5, the splenocytes, incubated

with no antigen were further restimulated for 5 hr with either no antigen or PMA and ionomycin. The splenocytes incubated with irradiated tumor were restimulated for 5 hr with irradiated tumor. The splenocytes were stained with anti-CD3-cychrome, anti-TNF-α-PE, and either CD8-FITC or CD69-FITC. As demonstrated in *Figure 10B,* 0.5% of tumor-stimulated, CD3+ splenocytes stained positive for both TNF-α and CD8. When CD3+ splenocytes were incubated in the presence of no antigen only (*Figure 10A*), 0.2% stained positive for TNF-α and CD8. This is a net increase of 0.3% which translates into a precursor frequency of 1 tumor-specific T cell in 333 CD3+ splenocytes. PMA/ionomycin treatment of the cells resulted in 4.9% of CD3+ T cells staining positive for TNF-α and CD8, shown as a positive control (*Figure 10C*). PMA-ionomycin, but not tumor also upregulated the number of CD3+ splenocytes staining positive for TNF-α and CD69, when compared to no antigen (*Figures 10D-10F*). These results indicate that a tumor-specific CD8 T cell population is elicited in response to tumor. These findings are important finding and will be investigated in future adoptive immuotherapy experiments.

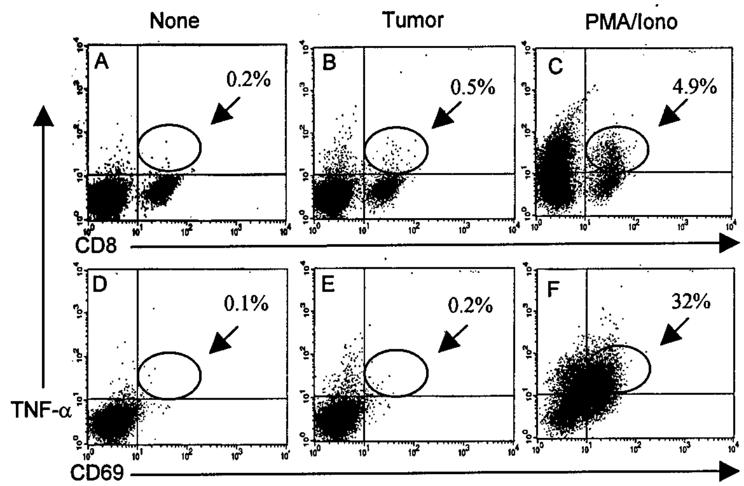


Figure 10. Tumor-reactive cytokine-secreting murine cells can be quantitated by FACS analysis. Shown is 3-color flow cytometric analysis of murine splenocytes stimulated with nothing (A and D), irradiated neu+ tumor cells (B and E) or PMA/ionomycin (C and F). Percentage of total CD3 cells is shown for the upper right quadrant, which represents cells dual stained for CD8 and TNF-α (panels A-C) or CD69 and TNF-α (panels D-F).

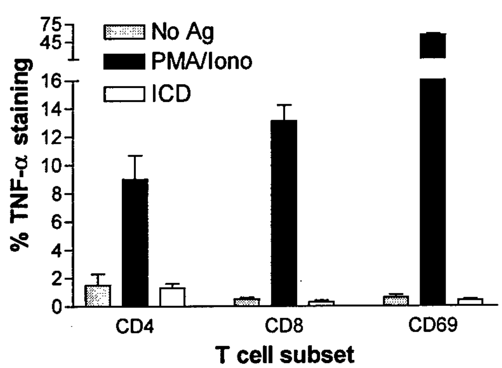


Figure 11. <u>Intracellular staining of mouse splenocytes after immunization with a neu ICD protein-based vaccine.</u> Neu-tg mice were immunized with a human neu ICD protein-based vaccine, ovalbumin (OVA) protein-based vaccine, or PBS alone at 14 day intervals for 2 immunizations in CFA. Data is expressed as the mean and standard deviation of 3 mice/group. PMA/ionomycin is used as a positive control for the assay. Mice from all three immunization groups were test for TNF-α production responses to PBS alone (white bar), 1 μg/ml human ICD protein (white bar), or PMA/ionomycin (black bar). Results are shown as the % of each T cell subset producing TNF-α as assessed by intracellular cytokine staining as described in Figure 10. Results shown are the mean of three mice (±sem). Similar results were observed in an independent experiment. Cells were dual stained with anti-CD4 and either CD45RO, HLA-DR, or CD69.

<u>K. Identification of neu-specific T cell subsets in neu-tg mice following vaccination with ICD protein-based assay.</u> Neu-tg mice were immunized 2 times, at 14-day intervals with recombinant human ICD. The splenocytes were isolated and incubated for intracellular staining as described in Figure 9. The results are presented graphically in Figure 11. The splenocytes stained were the same splenocytes tested for proliferative responses shown in Figure 6. I have also tested the splenocytes in ELIspot assay that also was unable to demonstrate cytokine release in response to antigen. The inability to measure cytokine release using this assay likely reflects a weakness in the assays. Measurement of intracellular cytokines using whole protein antigens has been problematic and processing of protein is not efficient (H. Maecker, personal communication). Recently, it has been shown that the introduction of the costimulatory antibodies to CD28 and CD49a into the stimulation can facilitate the detection protein-specific T cell precursors. Experiments are underway in our laboratory to optimize responses to protein antigen. Improving these techniques will also affect our ability to measure responses following DNA

vaccination.

L. Experiments in progress. In addition to optimizing measurement of antigen-specific precursors following vaccination, I also am conducting T cell infusion experiments following vaccination with whole protein ICD. I have, as outlined in the research protocol, mice currently being immunized with hICD protein. Fourteen days following the final vaccination, cells splenocytes will be isolated, T cell lines specific for either neu or ovalbumin (control) will be generated and infused into animals that had been previously implanted with NTT9-98 neu-expressing tumor cells described in Figures 1-3. The infusions will take place using graded doses of T cells to identify the number needed to eradicated established tumors (5 x 10^6 tumor cell implants). A similar experiment is also being carried out for the minimal disease model (5 x 10^5 tumor cell implants). Separation of CD4 and CD8 T cells is also being worked on in the laboratory for eventual use in identifying the numbers of CD8 or CD4 cells required to eradicate tumor.

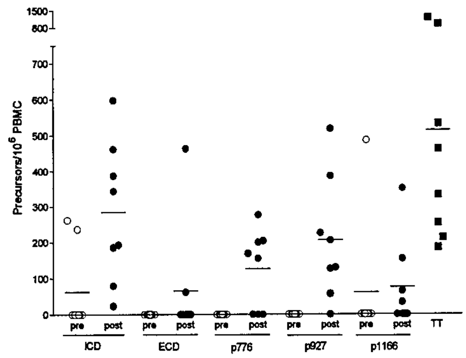


Figure 12. CD4 T cells specific for HER2 HLA-class II can be evaluated following *in vitro* stimulation by IFN-γ ELIspot. Data is shown as the pre- and post-immunizaiton IFN-γ T cell precursors for 8 patients who completed a HER2 peptide-based vaccine regimen. Pre-immunization precursor frequencies are shown as open circles and post-immunization precursor frequencies are shown as closed circles. Each symbol represents a unique patient and is the mean of sextuplet determinations. The ELIspot response to tetanus toxoid is shown as a positive control as black squares. In addition to testing pre- and post-immunization responses to the HER2 HLA-class II peptides (p776-790, p927-941, and p1166-1180), responses to the HER2 protein domains, ICD and ECD, were also examined. The mean value of each data set is depicted as a horizontal bar.

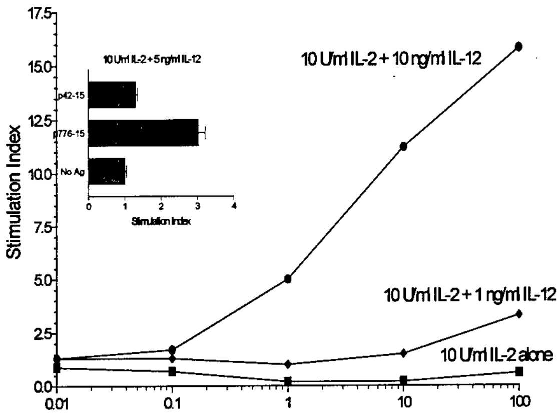


Figure 13. The addition of IL-12 to IL-2 during helper peptide stimulation results in increased antigen-specific proliferation. Proliferation data are shown following incubation of PBMC with varying concentrations of IL-12 in addition to IL-2 during peptide stimulation with HER2 peptide, p776-790. PBMC were derived from an HER2 immunized patient. The resulting PBMC were subsequently tested for peptide-specific proliferation testing varying concentrations (0-100 μg/ml) of p776-790 peptide (x-axis). The results are presented as a stimulation index as defined in Appendix B. Shown are the peptide-specific growth of cells carried through one in vitro stimulation with 10 Units/ml IL-2 (squares), 10 Units/ml IL-2 + 1 ng/ml IL-12 (diamonds), or 10 Units/ml IL-2 + 10 ng/ml IL-12. The inset shows that the proliferation response was specific for the stimulation peptide. Proliferation data is presented as the stimulation index. PBMC were incubated in the presence of IL-12 (5ng/ml), IL-2 (10 U/ml), and p776-790 peptide (10 ug/ml). The cells were examined for proliferation responses to no antigen, p776-790 (10 ug/ml), or p42-56 (10 ug/ml).

<u>II. Specific Aim B. To determine the feasibility of the expansion of HER2-specific CD4+ and CD8+ T cell lines from patients previously immunized with a HER2 peptide-based vaccine.</u>

A. <u>Validation of ELIspot assay to measure antigen-specific precursors following *in vitro* stimulation.</u> As previously described, I have developed an ELIspot assay to measure the peptide-specific precursors following *in vitro* stimulation. I have validated this assay for both HLA-class I peptide antigens from HER-2/neu (Figure 3 of Appendix B). This methodology is being directly applied to meet objectives of Specific Aim B. The results presented in Figure 3 of Appendix B demonstrate that precursors to CD8 HER2-specific T cells can be evaluated following *in vitro* stimulation. In another validation, I have assessed the application of this methodology to the measurement of CD4 HLA-class II restricted peptides. The PBMC used in this validation were isolated from patients undergoing a phase I clinical trial in which the patients were vaccinated with a peptide vaccine consisting of 3 HLA-class II peptides (p776-790, p927-941, and p1166-1180) derived from the ICD of HER2. As shown in Figure 12, HER2, HLA-class II peptide-specific precursors frequencies could be determined in patients prior to and following vaccination.

<u>B. IL-12, when added with IL-2 and peptide increased cell number, antigen-specific precursors, and antigen-specific function.</u>

One of the first tasks of this aim was to determine the whether IL-12, when added along with IL-2 during peptide stimulation will increase cell yield, antigen-specific function, and number of antigen-specific precursors. This work is near completion and mostly in manuscript form (Appendix C). The key findings are that IL-12 when added along with IL-2 during HLA-class I or class II peptide stimulation increases the cell yield following 1 or 2 IVS. The addition of IL-12 during peptide IVS with 9-mer peptide also results in increased 9-mer-specific T cells. IL-12 along with IL-2 also results in increased peptide-specific cytokine release from CD4 T cells. In addition to those findings we have been further examining the effects of IL-12, in combination with IL-2 on CD4 T cell function and antigen-specific expansion. The effects of IL-12 on the antigen-specific proliferation of CD4 T cells were examined. Cryopreserved PBMC from patient 1276 were incubated HER2 helper peptide (p776-790) either IL-2 alone or IL-2 in combination with IL-12. Following incubation the cells were tested for antigen-specific proliferation against various concentrations of p776-790. Figure 13 demonstrates that IL-12 dose-dependently increased the proliferation capacity, which suggests that IL-12 causes an increase in the number of peptide-specific T cells or increased blastogenic potential. This was answered using the ELIspot assay that was developed for helper peptides. As shown in Figure 14, IL-12 when added to IL-12 resulted an increase in p776-780-specific when compared with IL-2 alone.

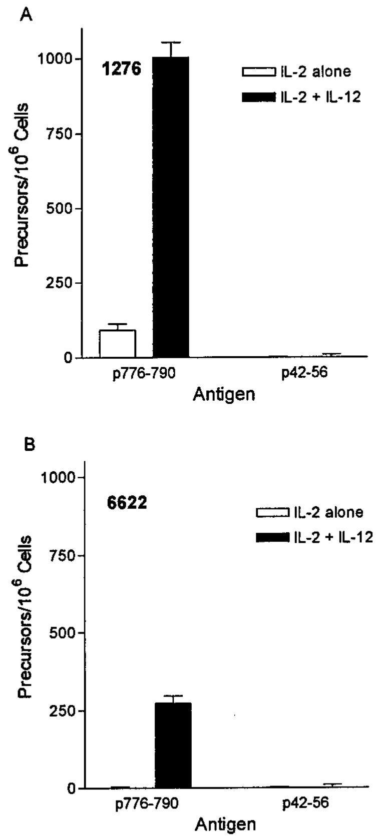
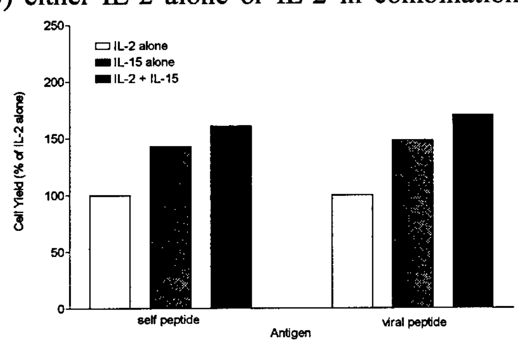


Figure 14. The addition of IL-12 to IL-2 during helper peptide stimulation results in increased antigen-specific T cell precursors. ELIspot data are shown following incubation of PBMC with 10 ng/ml of IL-12 in addition to 10 U/ml IL-2 during peptide stimulation with HER2 peptide, p776-790. PBMC were derived from HER2 immunized patients, 1276 and 6622. The resulting PBMC were subsequently tested for peptide-specific T cell precursors responding to 50 µg/ml p776-790 peptide. The results are presented as a precursors/million T cells. Results are presented as the mean (±sem) for 6 and 3 replicates for 6622 and 1276, respectively. Panel A shows the results for patient 1276 and Panel B shows the results for patient 6622. The cells were examined for either p776-790 (10 ug/ml), or an irrelevant helper p42-56 (10 ug/ml).



<u>**Figure 15: The addition of IL-15 to IL-2 increases total cell yield after IVS with HLA-A2 CTL peptides using PBMC derived from a cancer patient.**</u> Data shown is the increase in cell numbers for the patient donor, 9928, from cultures stimulated with 10 µg/ml of either an HLA-A2 self peptide, PAP pp13 or 10 µg/ml HLA-A2 viral peptide with 10 U/ml IL-2 alone (open bars), 20 ng/ml IL-15 (gray bars), or with 20 ng/ml IL-15 and 10 U/ml IL-2 (filled bars).

Notably, the precursor frequency was nearly 1:1000 cells for the cell line derived from patient 1276 and 1:4000 for the cell line derived from 6622. These findings and those included in Figure 13 are to be included in the manuscript in appendix C and submitted for publication.

C. IL-15 will increase total cell yield and increase surface expression of important activation and memory markers. Like IL-12, IL-15 enhances cell yield when used with IL-2 (Figure 15). As shown in Figure 16, IL-15 also enhances the expression of both memory (CD45RO) and early activation markers (CD69 and HLA-DR). These encouraging results have led me to continue examining IL-15. I have been provided IL-15 from Immunex Corporation (Seattle, WA) at no charge for these studies.

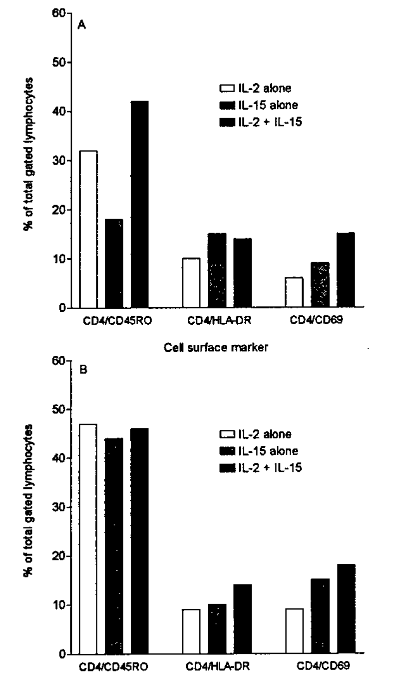


Figure 16: The addition of IL-15 to IL-2 increases memory and activation markers after IVS with either peptide or protein antigen. Flow cytometry data is shown from PBMC cultures stimulated with 10 μg/ml of either a helper tetanus toxoid peptide (panel A) or whole protein tetanus toxoid (panel B) along with 10 U/ml IL-2 alone (open bars), 20 ng/ml IL-15 (gray bars), or with 20 ng/ml IL-15 and 10 U/ml IL-2 (filled bars). Cells were dual stained with anti-CD4 and either CD45RO, HLA-DR, or CD69.

Key Research Accomplishments:

- A tumor cell line, NTT9-98, was established from neu-transgenic mice that is dose-dependently tumorigenic in neu-trangenic. This cell line was also found to immunogenic in the parental mouse strain, FVB/N. The important conclusion is that the doses of tumor cells can more accurately titrated for adoptive immunotherapy studies. In addition, this cell line maintains expression of both MHC class I and class II which will reduce the variable outcomes seen with progressive loss of both MHC class I and class II in spontaneous tumors.

- neu-tg mice can be immunized against neu with peptide and protein-based vaccines. (Manuscript published)

- neu DNA-vaccines were constructed in mammalian expression vectors.

- ELIspot was validated for both HLA-class I and class II epitopes. (Manuscripts published)

- IL-12 is beneficial when added to IL-2 during in vitro stimulation of peptide-specific T cells. IL-12 increases cell number, precursor frequency, and function of peptide-specific T cells. (Manuscript in preparation)

- IL-15 is increases cell number and activation markers on T cells following *in vitro* stimulation. This provides a basis for the continued studying the *in vitro* effects of the cytokine.

Reportable Outcomes:

Manuscripts published:

Knutson KL, Schiffmann K, and Disis ML, 2001, Generation of HER-2/neu CD8 T Cell Immunity in Cancer Patients Following Immunization with a HER-2/neu Helper Peptide Vaccine *Journal of Clinical Investigation* 107:477-484.

Knutson KL and Disis ML, 2001, Expansion of HER-2/neu Specific T Cells Ex Vivo Following Immunization with a HER-2/neu Peptide-based Vaccine. *Clinical Breast Cancer* 2:73-79.

Manuscripts submitted:

Knutson KL, Crosby PJ, and Disis ML, 2001, Isolation of HER2/neu (HER2) Specific T Cells Clones from an Ovarian Cancer Patient Following Immunization with a HER2 Peptide-based Vaccine. (Submitted to *The European Journal of Immunology*).

Abstracts published:

Knutson KL, and Disis, M.L., 2000 IL-12 Enhances In Vitro Expansion and Function of Peptide-Specific T Lymphocytes. Abstract #100790. *Proc. AACR* 41:193 (Award Winner, Abstract 1236).

Knutson KL, Crosby PJ, and Disis ML, 2000, Isolation of HER2/neu Specific γδTCR T cell Clones from a HER2/neu Immunized Ovarian Cancer Patient. *FASEB* 14:A1006 (Oral presentation, Abstract 54-13).

Disis M, Gooley T, **Knutson K**, Cheever M, Rinn K, Davis, and Schiffman K, 2000, A Phase I Study of Her-2/neu (Her2) Peptide-based Vaccines for the Generation of HER2 Specific Immunity in Patients with HER2 Overexpressing Cancers. *Proc. ASCO* 19:473A (Abstract 1857A).

Knutson KL, Crosby PJ, and Disis ML, 2000 Isolation of HER-2/neu (HER2) Specific T Cell Clones From an Ovarian Cancer Patient Following Immunization With a HER2 Peptide Vaccine. *Proc. Ovarian Cancer 2000* (Abstract).

Knutson KL, Schiffman K, and Disis ML, 2000 Generation of HER-2/neu (HER2) CD8+ T Cell Immunity in Cancer Patients Following Immunization with a HER2 Helper Peptide Vaccine. Abstract #. *Proc. AACR* 42:699 (Oral Presentation, Award Winner, Abstract 3758).

Conclusions:

Cancer vaccines are likely to be most effective for the prevention of relapse and possibly the prevention of new disease. Alternatively, adoptive T cell therapy is likely to be more effective for the treatment of established disease. Current problems of preventing the successful implementation of adoptive immunotherapy include (1) overcoming tolerance, (2) low numbers of tumor-specific T cells, (3) lack of definition of the immune effector arms most efficacious at tumor eradication, and (4) inability to adequately expand tumor-specific CD4 and CD8 T cells *ex vivo*. The current studies address all of these issues but are specifically designed to target identifying the immune effector arms required for the eradication of cancer and identifying appropriate techniques for successful *ex vivo* expansion of tumor-specific T cells.

The results presented show that we have immunization strategies (peptide-, protein-, and DNA-based vaccines) that are able to overcome tolerance in neu-tg mice. These vaccine strategies are currently being used to generate neu-specific T cell lines in vitro for the eventual goal of identifying the immune effector arms most effective at eradicating tumor. The findings of these experiments are likely to be readily translatable to expanding T cells from humans who have been vaccinated and further require tumor eradication using adoptive T cell therapy.

I have also presented evidence that IL-12 is effective, when added with IL-2, at increasing the number and function of tumor-specific T cells *in vitro*. These findings are currently in manuscript form and will be submitted in the near future. These findings are directly translatable to expanding human T cell lines that can be used for adoptive T cell therapy.

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Expansion of HER2/*neu*-Specific T Cells Ex Vivo Following Immunization with a HER2/*neu* Peptide-Based Vaccine

Keith L. Knutson and Mary L. Disis

Abstract

The identification and characterization of tumor antigens has facilitated the development of immune-based cancer prophylaxis and therapy. Cancer vaccines, like viral vaccines, may be effective in cancer prevention. Adoptive T-cell therapy, in contrast, may be more efficacious for the eradication of existing malignancies. Our group is examining the feasibility of antigen-specific adoptive T-cell therapy for the treatment of established cancer in the HER2/*neu* model. Transgenic mice overexpressing rat *neu* in mammary tissue develop malignancy, histologically similar to human HER2/*neu*-overexpressing breast cancer. These mice can be effectively immunized against a challenge with *neu*-positive tumor cells. Adoptive transfer of *neu*-specific T cells into tumor-bearing mice eradicates malignancy. Effective T-cell therapy relies on optimization of the ex vivo expansion of antigen-specific T cells. Two important elements of ex vivo antigen-specific T-cell growth that have been identified are (1) the preexisting levels of antigen-specific T cells and (2) the cytokine milieu used during ex vivo expansion of the T cells. Phase I clinical trials of HER2/*neu*-based peptide vaccination in human cancer patients have demonstrated that increased levels of HER2/*neu*-specific T cells can be elicited after active immunization. Initiating cultures with greater numbers of antigen-specific T cells facilitates expansion. In addition, cytokines, such as interleukin-12, when added during ex vivo culturing along with interleukin-2 can selectively expand antigen-specific T cells. Interleukin-12 also enhances antigen-specific functional measurements such as interferon-gamma and tumor necrosis factor-alpha release. Refinements in ex vivo expansion techniques may greatly improve the feasibility of tumor-antigen T-cell–based therapy for the treatment of advanced-stage HER2/*neu*-overexpressing breast malignancy.

Clinical Breast Cancer, Vol. 2, No. 1, 73-79, 2001

Key words: HER2/*neu*, Cancer vaccines, Adoptive T-cell therapy, Breast cancer, Cytokines

Introduction

The immunogenicity of tumors, such as breast cancer, reflects circumvention of tolerance to self-antigens. Increased understanding of how tolerance to self-antigens can be overcome has generated great interest in using immunotherapeutic approaches to prevent and eradicate breast malignancy.[1] One tumor-associated antigen in which there has been considerable interest is HER2/*neu*, a self-antigen overexpressed on many adenocarcinomas, including up to 30% of breast malignancies.[2,3] HER2/*neu* overexpression results from amplification and transcriptional upregulation of the *c-erbB-2/neu* proto-oncogene and is functionally important in tumor development and growth. In some subsets of breast cancer, HER2/*neu* overexpression correlates with a poorer prognosis.[4]

T-cell–mediated immunity directed against HER2/*neu* also exists in patients with HER2/*neu*-overexpressing cancer, suggesting that immunomodulatory intervention to enhance this immunity is possible.[5-7] Our laboratory is actively investigating the immunologic response to HER2/*neu* following vaccination with peptide-based vaccines.[8,9] In addition to the potential role of a vaccine in protection against subsequent tumor growth, these antigen-specific T cells could represent a source of cells for ex vivo expansion and ultimate use in adoptive T-cell–based immunotherapies targeting breast cancer (Figure 1).

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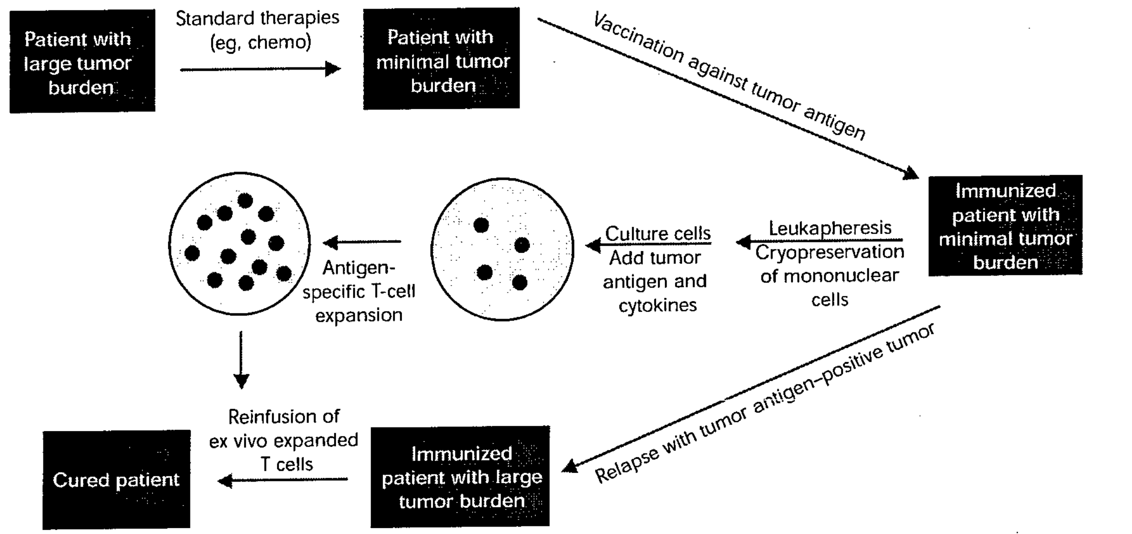
Figure 1 Hypothetical Model of Antigen-Specific Adoptive T-Cell Therapy



Methods

Immunization, Tumor Challenge, and Antigen-Specific Adoptive T-Cell Therapy in the neu-Transgenic Mouse Model

The intracellular domain (ICD) peptide-based vaccine consisted of the rat *neu* peptides spanning the amino acid positions 781-795, 788-802, 932-948, and 1171-1185. These peptides were predicted to elicit *neu*-specific T-cell immunity.[10] The *neu*-transgenic (*neu*-Tg) mice were immunized with peptides at a final concentration of 100 μg each in a total volume of 200 μL, including adjuvant. The animals underwent two immunizations 14-16 days apart. Control animals received adjuvant alone. Mice were challenged with tumor at indicated times using subcutaneous injection of 2 x 10^6 purified syngeneic tumor cells derived from spontaneously occurring mammary tumors. T cells used for adoptive transfer were derived from spleen of control mice or mice that had been previously vaccinated with the ICD peptide-based vaccine. These T cells were either used directly or cultured for 10 days in the presence of the same peptides used for vaccination with periodic additions of interleukin-2 (IL-2).

Patient Vaccinations

Patients were enrolled into a Food and Drug Administration–approved phase I clinical trial evaluating the toxicity and immunogenicity of a HER2/*neu* peptide-based vaccine, as previously described.[9] Briefly, the patients were immunized monthly for 6 months with a HER2/*neu* peptide vaccine consisting of putative HER2/*neu* peptides spanning the amino acids 369-384, 688-703, and 971-984. Each peptide contained within its sequences the human leukocyte antigen (HLA)-A2 binding peptides, p369-377, p689-697, and p971-979, respectively.

Generation and Expansion of Human T-Cell Lines and Clones

Antigen-specific T-cell lines and clones were generated by culturing patient peripheral blood mononuclear cells (PBMC) in the presence of HER2/*neu* peptide, as previously described.[9] Briefly, for the generation and expansion of HER2/*neu*-specific T-cell lines, PBMCs were cultured in 1 μM each of the HER2/*neu* HLA-A2 peptide, p369-377. For cloning, the T-cell lines were diluted to achieve approximately 0.3 viable cells/200 μL and plated onto 96-well plates in culture medium. Peptide-pulsed, irradiated autologous PBMCs (2.0 x 10^5) were added to each well in the presence of IL-2. The clones were eventually expanded and carried using IL-2 and peptide-pulsed, irradiated autologous Epstein-Barr virus (EBV)-transformed B-lymphoblastic cells (BLCL).

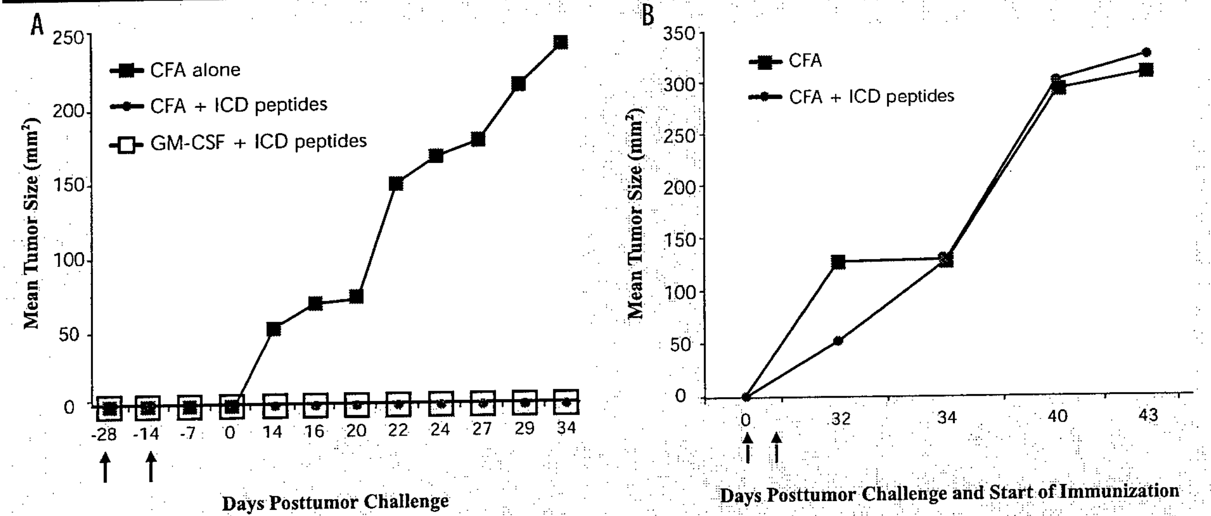
Chromium-Release Assays

Cytolytic activity was measured using standard chromium-release assays. Targets used were peptide-pulsed BLCLs, which were labeled with chromium-51 for 1-2 hours prior to use. Killing of target cells is detected as the release of chromium-51. The cytolytic reaction was carried out for 4 hours at 37°C, after which the amount of chromium-51 released into the cell culture media was assessed.

Enzyme-Linked Immunosorbent Spot

An enzyme-linked immunosorbent spot (ELIspot) assay was used to determine frequencies of peptide-specific CD8 T

Figure 2 *Neu* Peptide Immunization Protects Against Tumor Challenge but Is Ineffective in Mediating Regression of Existing Disease



Panel A represents data from *neu*-transgenic mice immunized with a *neu* intracellular domain (ICD) peptide-based vaccine twice at 14-day intervals (arrows) prior to subcutaneous injection on day 0 of 2 x 10^6 syngeneic *neu*-overexpressing tumor cells. Regardless of adjuvant, granulocyte-macrophage colony-stimulating factor (GM-CSF), or Complete Freund's Adjuvant (CFA), all animals rejected tumor (5 mice/group). The CFA + ICD peptides and the GM-CSF + ICD peptides curves overlap completely at zero. **Panel B** represents data from animals that received 2 x 10^6 tumor cells on the same day immunizations with *neu* ICD peptides were initiated (day 0). Despite two immunizations, at 14-day intervals (arrows; day 0 and day 14), mice were unable to reject tumor. Both panels show data as the mean of tumor dimensions.

lymphocytes, as previously described.[9,11] T cells are activated in vitro by being cocultured with antigen and plated onto nitrocellulose 96-well plates coated with anti-interferon-gamma antibody. The cells are incubated at 37°C for 24 hours and the interferon-gamma (IFN-γ) locally released during the incubation period is captured nearby the cell. Following the incubation period, the captured IFN-γ is detected with another secondary antibody. Binding of the secondary antibody is detected colorimetrically, and the resulting spots, representing individual cells, are enumerated. The number of antigen-specific spots is determined by subtracting the spots obtained in no antigen wells from the number of spots obtained in the presence of antigen.

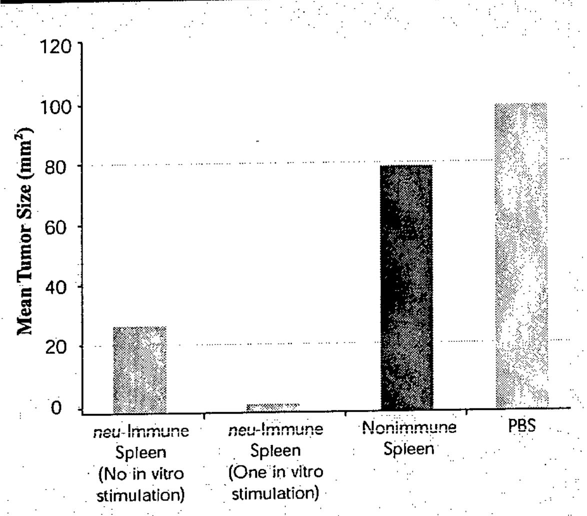
Differences in the Clinical Role of Cancer Vaccines vs. Adoptive T-Cell Therapy

It is our view that the major role of cancer vaccines likely lies in the cancer prevention rather than as a treatment modality in eradicating established malignancy. This is analogous to viral vaccines, which are administered as a prophylactic to prevent disease (eg, influenza) but not to cure it. This observation can be illustrated in an animal model of breast cancer. The *neu*-Tg mouse is an FVB mouse strain genetically engineered to overexpress rat *neu* specifically in mammary tissue.[12] Female mice spontaneously develop mammary tumors after a long latency that histologically resembles human breast carcinoma, including *neu* overexpression. In these transgenic mice, rat *neu* is a self-antigen. In previous studies, we identified several putative major histocompatibility complex (MHC) class II binding helper peptides of rat *neu*[10] and found that immunization of rats with *neu* peptides elicited *neu*-specific T-cell immunity in vivo. These peptides were then used for immunization in the *neu*-Tg mouse model, where it was also observed that mice developed *neu*-specific T-cell responses. In tumor protection studies, we observed that two vaccinations with *neu* peptides, derived from the ICD, were adequate for efficient rejection of tumor cells implanted subcutaneously after immunization (Figure 2A). In contrast, the mice were unable to reject tumor if vaccination was begun concurrently with tumor inoculation (Figure 2B). Thus, vaccination protects *neu*-Tg mice against future tumor challenge.

The primary purpose of adoptive T-cell therapy is to augment T-cell responses over and above that achievable by vaccination alone.[13] Vaccination itself can increase the number of immune T cells capable of recognizing and responding to antigen. While repeated vaccination increases the number of immune effectors, eventually a plateau is reached after which further immunizations have little to no impact in further increasing the number of tumor-specific T cells.[13] Adoptive T-cell therapy allows levels of immunity to be achieved that could potentially mediate an antitumor response, and adoptive transfer of T cells can result in the infused cells representing a large fraction (eg, 1/2) of the host's lymphocytes.[13]

Although the efficacy of administering high numbers of T cells to treat cancer in animal models is well established, in humans such attempts have met with limited success. Cancers that have been successfully treated by adoptive T-cell transfer include the EBV-related disorders, immunoblas-

Figure 3 Adoptive Transfer of *neu*-Specific T Cells Inhibits Growth of *neu*-Overexpressing Tumors In Vivo



Mice were immunized with a *neu* intracellular domain (ICD) peptide-based vaccine two times 14 days apart. Spleens were harvested from immune animals and infused (no in vitro stimulation [IVS]) into tumor-bearing mice or enriched with one IVS and then infused (one IVS). Mice were implanted with 2 x 10^6 *neu* syngeneic tumor cells on day 1, and on day 2, mice were infused with 2 x 10^7 splenocytes derived from immune spleen, immune spleen with one IVS, spleen from nonimmunized syngeneic mice, or PBS alone. Data are the mean of tumor dimensions from 3 mice/group measured 30 days after implant.

tic lymphoma, and Hodgkin's disease.[14-16] In those studies, EBV-specific cytotoxic T lymphocytes (CTL) generated ex vivo and subsequently reinfused into patients were effective in both prophylaxis and the treatment of disease. Furthermore, the infused CTLs persisted for long periods while maintaining their antiviral properties. However, the major distinction between the treatment of EBV malignancies and nonviral cancers is the presence of nonself viral antigens. In nonviral cancers, tolerance-mediated deletion of potent and robust T cells has occurred to some extent and makes the ex vivo generation of an adequate antigen-specific T-cell population a challenge. One way to overcome this challenge is to vaccinate against antigen before retrieving T cells from the patient for expansion. Vaccination can increase the number of antigen-specific T cells in vivo.

To understand more fully eradication of HER2/*neu*-overexpressing breast cancer by adoptive T-cell therapy, we initiated studies using the *neu*-Tg mice. The data suggest that the transfer of *neu*-specific splenocytes from *neu* peptide-immunized *neu*-Tg mice into nonimmunized naive mice can eradicate preestablished *neu*-overexpressing mammary tumors (Figure 3). The ability of the splenocytes to kill tumor was greatly enhanced by increasing the number of tumor-specific T cells with cell culture (ie, ex vivo enrichment) prior to reinfusion. In contrast, splenocytes derived from naive *neu*-Tg mice were unable to prevent tumor growth, demonstrating that prior immunization of the animals was necessary to mediate the response. Therefore, a hurdle to overcome for human clinical trials of adoptive T-cell therapy is the development of techniques for ex vivo expansion of HER2/*neu*-specific T cells.

Ex Vivo Expansion of Antigen-Specific T Cells

The requirements for the ex vivo expansion of T cells that would allow the generation of a maximal number of antigen-specific T cells while retaining optimal antigen-specific function are not well understood. Two of the most important obstacles are (1) a low preexisting antigen-specific T-cell precursor population and (2) lack of an appropriate in vitro cytokine environment.[13]

The preexisting systemic level of antigen-specific T cells is important in culturing T cells. In unprimed individuals, the ex vivo expansion of HER2/*neu* antigen-specific T cells from peripheral blood is difficult because levels of these antigen-specific T cells can be extremely low. For example, isolation and expansion of HER2/*neu*-specific T cells from patients with HER2/*neu*-overexpressing breast or ovarian carcinomas is laborious and involves lengthy expansion techniques with multiple in vitro stimulations.[6] Therefore, in order to establish T-cell lines of sufficient antigen-specificity from unprimed individuals, one would have to start by culturing very large numbers of PBMCs.[17]

One strategy to improve expansion is to increase the frequency of antigen-specific precursors in vivo prior to ex vivo

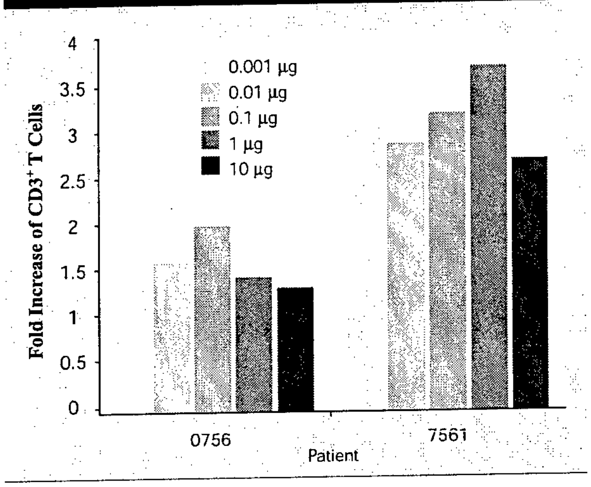
Table 1 HER2/*neu* Peptide Immunization Results in Increased HER2/*neu* T-Cell Precursors

Donor	Preimmunization p369-377 9-mer	Postimmunization p369-377 9-mer	pflu-9-mer	TT
HLA-A2 Volunteer Donor	< 1:100,000	Not done	1:10,000	1:9000
Patient 2859	< 1:100,000	1:3800	< 1:100,000	1:22,000
Patient 0107	< 1:100,000	1:12,000	1:4500	1:4500

Patients 2859 and 0107 (both HLA-A2⁺) received the HLA-A2 vaccine containing three HER2/*neu* T-helper epitopes, p369-384, p688-703, and p971-984. P369-384 contains the HLA-A2 motif, p369-377 9-mer. Pflu-9-mer is an HLA-A2 binding motif contained within the influenza matrix protein. Precursor frequencies for an HLA-A2⁺ volunteer donor are shown for comparison. Results are presented as the ratio of p369-377 9-mer–specific T-cell precursors per number of peripheral blood mononuclear cells.

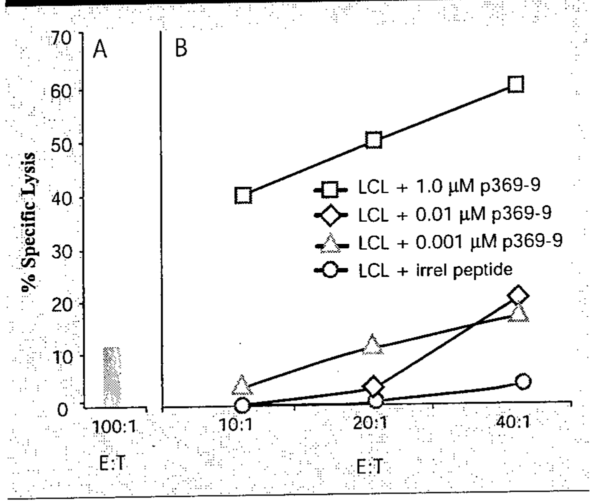
Abbreviation: HLA = human leukocyte antigen

Figure 4 Concentration of HER2/*neu* Peptide p369-377 9-mer Used in In Vitro Stimulation



T lymphocytes can be expanded ex vivo through two in vitro stimulations with peptide antigens. Data are shown from two patients who completed a course of six immunizations with a HER2/*neu* peptide-based vaccine. Both patients were HLA-A2^{+} and responded predominantly to p369-384 15-mer, which contains the HLA-A2 epitope, p369-377 9-mer. Cultures of peripheral blood mononuclear cells were taken through two in vitro stimulations with various concentrations of p369-377 9-mer. The graphs represent expansion of total CD3^{+} T cells over starting numbers.

Figure 5 Lytic Activity of Antigen-Specific T Cells Is Enriched Following Two In Vitro Stimulations



Data shown are from patient 7561, who completed a course of six immunizations with a HER2/*neu* peptide-based vaccine. The dominant response was directed against p369-384 15-mer, which contains the HLA-A2 epitope, p369-377 9-mer (p369-9). The patient was HLA-A2^{+}. **Panel A** demonstrates the level of lytic activity of T cells after one in vitro stimulation (IVS) with p369-377 9-mer. The lytic activity was examined at an effector-to-target (E:T) ratio of 100:1 against autologous, chromium-51–labeled Epstein-Barr virus–transformed lymphoblastoid cell line (LCL) loaded with p369-377 9-mer. Activity against nonloaded LCL was < 1%. **Panel B** demonstrates enrichment of lytic activity of T cells after two IVS with p369-377 9-mer. Target LCL were loaded with varying concentrations of peptides. Cultures from both panels were established by IVS with 1 µM p369-384 15-mer (immunizing peptide).
Abbreviation: irrel peptide = irrelevant HLA-A2 binding peptide

expansion by vaccination. Studies from our group have shown that boosting HER2/*neu* precursor frequencies by peptide immunization has allowed us to more readily expand and clone HER2/*neu*-specific T cells from the peripheral blood of patients with HER2/*neu*-overexpressing breast cancers as compared to naive donors (K. Knutson, unpublished observations).

Phase I clinical trials were initiated in 1997 to assess both toxicity and immunogenicity of HER2/*neu* peptide-based vaccines in patients with stage III or IV breast or ovarian cancer. All of the vaccine formulations target HER2/*neu* peptides in either the ICD, extracellular domain (ECD), or both.[2] Granulocyte-macrophage colony-stimulating factor was used as an adjuvant,[18] and the immunizations were administered intradermally monthly for 6 months. The initial trial tested three helper T-cell vaccines, each composed of three MHC class II binding peptides, 15-18 amino acids in length.[2,9] One of the vaccine preparations (HLA-A2 vaccine) consisted of three putative T-helper epitopes of HER2/*neu*, each of which contains a complete HLA-A2 binding motif within their sequence. Vaccines using this strategy have been demonstrated to elicit both helper and cytolytic T-cell immunity.[9]

Recently, the analysis of the 19 patients who received the HLA-A2 vaccine was reported.[9] In that study, it was observed that the majority of patients developed both HER2/*neu* peptide- and protein-specific immunity. Despite generating significant levels of immunity, the HER2/*neu* vaccine was well tolerated. Detection of HER2/*neu*-specific T cells was assessed using an IFN-γ–ELIspot assay, which has been shown to be a reliable method to detect antigen-specific T cells existing in the peripheral blood at levels as low as 1 in 100,000 mononuclear cells. We demonstrated that T cells specific for HER2/*neu* were increased following vaccination[9] (Table 1), allowing us to more readily expand functional T cells in the presence of antigen (Figure 4).

The antigen-specific expansion of the T cells also enriches the cytolytic activity against HER2/*neu* (Figure 5). Furthermore, increased T-cell precursors facilitate isolation of T-cell clones. For example, we isolated 21 p369-377 peptide-specific clones from an HLA-A2 patient immunized with the HLA-A2 vaccine.[19] Most of the clones (19 of 21) expressed the broad T-cell marker, CD3. While 11 of the clones expressed CD8+ (cytolytic T cells), nine expressed CD4+ (helper T cells) despite being cloned with the HLA-A2–binding 9-mer. Since 9-mer peptides in association with HLA-A predominantly regulate CD8+ T cells, the significance of CD4+ T cells responding to HLA-A2 9-mer is not well understood. Also, 19 of the clones expressed the alpha-beta T-cell receptor, and interestingly, two expressed the gamma-delta T-cell receptor.[20] Additionally, several of the clones secreted IFN-γ directly in response to p369-377 9-mer and could lyse HLA-A2+, HER2/*neu*-overexpressing tumor cells. These findings suggest that vaccination may have the potential to expand different HER2/*neu*-specific T-cell subsets and may eventually allow identification of T cells most effective at eradicat-

Table 2 Cytokines Used for Ex Vivo Expansion of Antigen-Specific T Cells

Cytokine	Natural Source	Function
IL-2	Activated T cells	Proliferation of T cells[13]
IL-12	Phagocytes	Proliferation of T cells[22]
		Enhances cytolytic activity[23]
		Promotes cell-mediated immunity[24]
		Enhances cytokine production[25]
		Upregulates IL-2 receptor expression[26]
IL-4	CD4+ T cells	Enhances IL-12 production[27]
		Enhances maturation of memory T cells[28]
		T-cell differentiation[29]
IL-7	Stromal cells	T-cell survival[30]
		T-cell proliferation[31]
IL-15	Activated T cells	Proliferation of T cells[32]
		Maintenance of memory T cells[33]
		Inhibition of apoptosis[34]

Abbreviation: IL = interleukin

ing tumor.

The cytokine environment needed to expand a functional antitumor population and sustain it in vivo has been elucidated to some degree.[13] For example, IL-2, when added during in vitro stimulation of T cells, promotes proliferation and increased survivability of cells in vivo.[13] However, IL-2 activates T-cell lines nonspecifically and often antigen specificity is not preserved. We have found that IL-12, when added along with IL-2, enhances both expansion and function of HER2/*neu*-specific T-cell lines.[21] For example, IL-12 plus IL-2 enhances HER2/*neu*-specific tumor necrosis factor-alpha release up to sixfold greater than that observed with IL-2 only following in vitro stimulation with HER2/*neu* peptides. Over the past decade, other T-cell–active cytokines, such as IL-4, IL-7, and IL-15, have been identified, which may exhibit similar in vitro activities (Table 2).[13,22-34] Studies are ongoing in our laboratory to characterize these cytokines for their utility in ex vivo expansion of HER2/*neu*-specific T cells.

Conclusions

Tumor antigen-targeted T-cell–based therapy is a feasible treatment strategy for HER2/*neu*-overexpressing malignancies. Vaccination alone most likely will not be effective as a treatment strategy for established disease but rather in preventing the recurrence of micrometastatic disease. Treatment of established disease will require T-cell therapy with ex vivo–expanded tumor-specific T cells. The use of vaccination followed by carefully manipulated ex vivo antigen-mediated expansion of T cells offers the potential of increasing tumor-specific T cells to numbers greater than that which could be achieved by vaccination alone. Ex vivo expansion has been problematic and many hurdles will need to be overcome. Two important issues are (1) increasing antigen-primed T cells in vivo prior to ex vivo expansion by vaccination and (2) improved culture conditions with the use of recently identified cytokines in conjunction with IL-2. The refinement of techniques for antigen-specific T-cell therapy has ushered in a new era for the treatment of breast cancer.

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Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients

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CD4 T-cell help is required during the generation and maintenance of effective antitumor CD8 T cell–mediated immunity. The goal of this study was to determine whether HER-2/neu–specific CD8 T-cell immunity could be elicited using HER-2/neu–derived MHC class II "helper" peptides, which contain encompassed HLA-A2–binding motifs. Nineteen HLA-A2 patients with HER-2/neu–overexpressing cancers received a vaccine preparation consisting of putative HER-2/neu helper peptides p369–384, p688–703, and p971–984. Contained within these sequences are the HLA-A2–binding motifs p369–377, p689–697, and p971–979. After vaccination, the mean peptide-specific T-cell precursor frequency to the HLA-A2 peptides increased in the majority of patients. In addition, the peptide-specific T cells were able to lyse tumors. The responses were long-lived and detectable for more than 1 year after the final vaccination in select patients. These results demonstrate that HER-2/neu MHC class II epitopes containing encompassed MHC class I epitopes are able to induce long-lasting HER-2–specific IFN-γ–producing CD8 T cells.

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Introduction

The cytolytic CD8 T cell is generally thought to be the major mediator of antitumor immunity. Many tumor antigens have been discovered within the last decade, from which multiple MHC class I–restricted epitopes have been identified (1). One such tumor antigen that is overexpressed on several cancers, including breast and ovarian cancers, is HER-2/neu, the gene product of *erbB2/neu* proto-oncogene (2–4). Using both predictive and MHC elution techniques, it has been possible to identify several MHC class I–restricted epitopes of HER-2/neu for use in immune-based cancer therapies (5–7).

Antitumor immunization strategies have taken many forms, including the use of whole cell-, peptide-, protein-, and DNA-based vaccines. Peptide-based vaccines are attractive over other forms because peptides are (a) easily constructed, (b) chemically stable, (c) free of contaminating substances such as bacterial pathogens, and (d) devoid of oncogenic potential. Although some successful immunization has been achieved using MHC class I–restricted peptide-based vaccines (8, 9), many studies with native, unmodified peptide resulted in no response or only low-level responses (10–13). For example, Pass and colleagues demonstrated generation of peptide-specific precursors to the gp100$_{209-217}$ in five of six melanoma patients following immunization (8). In contrast, in parallel studies from the same group it was observed that only two of seven and zero of seven patients had detectable precursors to gp100$_{280-288}$ or MART-1$_{27-35}$, respectively, after immunization (8). Immunity to MHC class I peptides can be augmented by adding "help" in the form of CD4 T-helper cells. CD4 T-cell responses are essential, both in order to extend the life of the antitumor CD8 T cells and to promote the accumulation of antigen-presenting cells at the tumor site (14).

The necessity of CD4 T-cell help to generate and sustain the MHC class I–restricted CD8 T-cell responses has led to the use of universal, nonspecific MHC class II–restricted epitopes such as PADRE in clinical vaccination trials (15, 16). Although responses to the universal MHC class II–restricted epitopes are typically increased, the responses to the tumor antigen epitopes usually have been limited. We have hypothesized that increased immunogenicity to MHC class I–restricted epitopes may be achieved by immunizing with MHC class II–restricted epitopes derived from the same protein (12, 17).

In this study we evaluated whether active immunization with HER-2/neu helper peptide epitopes, each containing putative HLA-A2 MHC class I epitopes, would generate both CD4 and CD8 T-cell peptide and protein responses in vivo. In addition, we questioned whether HER-2/neu peptide–specific T cells, if they could be elicited, could recognize naturally processed and presented tumors. Finally, we questioned at what level immunity is elicited and how long immunity would last, as the ultimate goal is to generate long-term protective immunity against de novo formation or recurrence of tumor.

Methods

Clinical trial. Between August 1996 and August 1998, 19 patients with breast or ovarian cancer were enrolled in a phase I HER-2/neu peptide-based vaccine trial approved by the University of Washington's Human Subjects Division and the United States Food and Drug Administration. Eligibility was dependent upon subjects (a) being diagnosed with stage III/IV breast or ovarian cancer and having been treated for their primary and metastatic disease according to recommended disease-appropriate standards with surgery, chemotherapy, radiation therapy, or combined modality, (b) having a white blood cell count greater than 3.5 dl/ml, (c) showing HER-2/neu protein overexpression in the primary tumor or metastasis, (d) being off immunosuppressive drugs and chemotherapy for at least 30 days before enrolling, and (e) being HLA-A2 positive. Patients were tested for immune competence responsiveness to a minimum of two of seven recall antigens by skin testing with Multitest CMI (Pasteur Merieux Connaught Labs, Institut Merieux, Lyon, France). All patients signed a protocol-specific consent and received monthly vaccinations with three 15-amino acid (15-aa) HER-2/neu–derived peptides, p369–p384, p688–p703, and p971–p984, containing within each the putative HLA-A2–binding motifs p369–p377 (6), p689–p697 (7), and p971–p979 (18). Five hundred micrograms of each peptide (1.5 μg total peptide dose) were solubilized in 10 mM sodium acetate (pH 4.0) and admixed with 125 μg rhuGM-CSF (kindly supplied by Immunex Corp., Seattle, Washington, USA) as an adjuvant. The vaccine preparation was divided into two intradermal injections administered to the same draining lymph node site monthly for 6 months. Subjects underwent peripheral blood draws or a leukapheresis before and 30 days after each vaccination for immunologic monitoring.

Materials. The following peptides used in this study, either for immunization or in vitro use, were HLA-A2 flu matrix peptide (pFlu), GILGFVFTL (19); HLA-A2 cytomegalovirus (CMV) peptide, NLVPMVATV (20); and HER-2/neu peptides, p369-384, KIFGSLAFLPESFDGDPA (21), p688-703, RRLLQETELVEPLTPS (21), p971-984, ELVSEFSRMARDPQ (21), p369-377, KIFGSLAFL (6), p689-697, RLLQETELV (7), and p971-979, ELVSEFSRM (18). All peptides used for in vitro immunological monitoring were manufactured either by United Biochemical Inc. (Seattle, Washington, USA) or Multiple Peptide Systems Inc. (San Diego, California, USA), and all were greater than 95% pure as assessed by HPLC and mass-spectrometric analysis. Peptides used in vaccine preparations were manufactured by Multiple Peptide Systems (kindly provided by Corixa Corp., Seattle, Washington, USA) and approved for use in humans. Ficoll-Hypaque was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). RPMI-1640, HBSS, and PBS were purchased from Life Technologies (Rockville, Maryland, USA) and EHAA-120 from Biofluids (Rockville, Maryland, USA). [^{3}H] thymidine and [^{51}Cr] sodium chromate were purchased from NEN Life Science Products Inc. (Boston, Massachusetts, USA), human AB+ serum from Valley Biomedical Inc. (Winchester, Virginia, USA), sterile nitrocellulose-backed microfiltration 96-well plates from Millipore Corp. (Bedford, Massachusetts, USA), and streptavidin-alkaline phosphatase and AP-colorimetric reagents were from Bio-Rad Laboratories Inc. (Hercules, California, USA). Purified anti–IFN-γ (clone number 1-D1K) and biotin-conjugated anti–IFN-γ (clone number 7-B6-1) were purchased from Mabtech AB (Nacka, Sweden). Recombinant HER-2/neu protein domains (intracellular domain [ICD] and extracellular domain [ECD]) were provided by Corixa Corp. HLA testing was performed by the Puget Sound Blood Bank (Seattle, Washington, USA).

Cell lines. Epstein-Barr virus–transformed (EBV-transformed) lymphoblastoid cells (BLCLs) were produced from PBMCs using culture supernatant from the EBV-producing B95-8 cell line (American Type Culture Collection, Manassas, Virginia, USA). HLA-A2$^+$ BLCLs stably expressing human HER-2/neu were a kind gift from Steve Fling of Corixa Corp. The HER-2/neu–overexpressing cell lines SKOV3 and SKOV3-A2 and BLCLs were maintained in RPMI-1640 with L-glutamine, penicillin, streptomycin, 2-mercaptoethanol, and 10% FCS. The SKOV3-A2 tumor cells are the same as SKOV3 tumor cells, except they are stably transfected with a vector encoding HLA-A2 (22).

Preparation of PBMCs. PBMCs were obtained either by leukapheresis or 180–250 ml blood draws and isolated by density gradient centrifugation as described previously (21). Cells were analyzed immediately or aliquoted and cryopreserved in liquid nitrogen in freezing media (90% FBS and 10% dimethylsulfoxide) at a cell density of 25–50 × 10^6 cells/ml.

T-cell proliferation assays. HER-2/neu–specific T-cell proliferative responses were measured at base line, before each vaccination, and at the end of the study. T-cell proliferation was assessed using a modified limiting dilution assay designed for detecting low-frequency lymphocyte precursors based on Poisson distribution as described previously (21, 23). Data is reported as a stimulation index (SI), which is the mean of 24 experimental wells/mean of 24 no-antigen wells. An age-matched control population of 30 volunteer blood donors was analyzed similarly (data not shown). No volunteer donor had a response to HER-2/neu proteins or peptides. The mean and 3 SDs of the volunteer donor responses to all antigens (SI of 1.98) established a base line, therefore an SI greater than two was considered consistent with an immunized response.

Enzyme-linked immunosorbent spot assay. A 10-day enzyme-linked immunosorbent spot (ELIspot) assay was used to determine precursor frequencies of peptide-specific CD8 T lymphocytes as described previously (24). Briefly, on day 1, 2.5 ×10^5 PBMCs/well were plated into 96-well plates in six-well replicates in 200 μl of RPMI-1640 containing L-glutamine, penicillin, streptomycin, and 10% AB serum (T-cell medium) in the presence or absence of 10 μg/ml peptide antigen or 0.5 U/ml tetanus toxoid. The cells were incubated at 37°C at 5% CO_2. On day 5, IL-2 was added to 10 U/ml. On day 8, 2.5×10^5/well irradiated autologous PBMCs and 10 μg/ml antigens were added. Also on day 8, nitrocellulose-backed 96-well plates (NC-plates) were coated with 10 μg/ml anti–IFN-γ Ab in PBS at 50 μl/well. On day 9 the NC-plate was washed three times with PBS and blocked for 2 hours with PBS containing 2% BSA, followed by three washes with PBS. On day 9, the cells were gently resuspended, pooled, centrifuged, and the media was replaced. The cells were transferred into the NC-plate in a volume of 100 μl/well in T-cell medium. The NC-plate was incubated at 37°C for a further 20–24 hours followed by washing three times using PBS containing 0.05% Tween-20. The plate was then incubated for 2.5 hours at room temperature in 50 μl/well PBS containing 5 μg/ml biotinylated anti–IFN-γ Ab, washed three times with PBS, and further incubated with 100 μl/well streptavidin-alkaline phosphatase at a dilution of 1:1,000 in PBS for 2 hours at room temperature. After washing three times in PBS, the plate was incubated with 100 μl/well AP-colorimetric substrate for 20–30 minutes, rinsed with cool tap water, and allowed to dry completely. Resultant spots were then enumerated using a dissecting microscope. Precursor frequencies were calculated by subtracting the mean number of spots obtained from the no-antigen control wells from the mean number obtained in the experimental wells. Statistical analysis was performed using the Student's t test (Microsoft Excel 97).

Precursor frequencies to viral peptide antigens were also enumerated from peripheral blood from four HLA-A2$^+$ healthy, volunteer individuals for comparison purposes. Assay validation was established in preliminary studies using the HLA-A2, pFlu peptide over a PBMC range of $1.0–3.5 \times 10^5$ cells and also with the use of IFN-γ–coated polystyrene beads (24). These preliminary studies demonstrated that the assay is linear and precise between 2.0 and 3.5×10^5 PBMCs/well, has a detection limit of 1:100,000, and has a detection efficiency of 93%. The attributes of this assay, such as the limit of detection, are consistent with previously reported ELIspot methods (25, 26). The background number of spots per well, in the absence of antigen, was 10 ± 1 (mean ± SEM, $n = 180$). A positive response was defined as a precursor frequency that was both significantly ($P < 0.05$) greater than the mean of control no-antigen wells and detectable (i.e., >1:100,000). Although the ELIspot assay is sensitive and suitable for detecting low-level responses to vaccination (8, 13, 25), it is currently unknown if the calculated precursor frequencies represent actual numbers of antigen-specific cytolytic T cells in the peripheral blood.

Generation of antigen-specific T-cell lines and clones. Antigen-specific T-cell lines and clones were generated by culturing 25×10^6 PBMCs in T25 tissue-culture flasks in 20 ml of T-cell medium. For the generation of HER-2/neu–specific T-cell lines, PBMCs were cultured in 1 μM each of the HER-2/neu 9-aa peptides, p369–377, p689–697, and p971–979. For generation of p369–377-specific clones, p369–377 peptide was added to the flasks to 1 μm. The flasks were incubated at 37°C and 5% CO_2. On day 3 and every other subsequent day, IL-2 was added to 5 U/ml. On day 10, in vitro stimulation (IVS) was performed with peptide-pulsed, irradiated autologous PBMCs. The cultures were further incubated for an additional 10 days with periodic IL-2 administration. After the second IVS, the antigen-specific T-cell lines were examined for cytolytic activity as described below and in some cases were cloned. For cloning, bulk cultures were diluted to achieve approximately 0.3 viable cells/200 μl and plated onto flat-bottom 96-well plates in complete medium. Peptide-pulsed, irradiated autologous PBMCs (2.0×10^5) were added to each well in the presence of 50 U/ml IL-2. The wells were then tested for lytic activity in a [^{51}Cr]-release assay using 50 μl of cells from each well after 14 days. Positive wells were identified as those having specific activity of 5% or greater. The positive wells were transferred to new 96-well plates and subsequently restimulated with peptide-pulsed, irradiated autologous BLCLs. The cultures were eventually expanded and carried using IL-2 and peptide-pulsed, irradiated autologous BLCLs.

*[^{51}Cr]-release assays.*C ytolytic activity was measured using standard [^{51}Cr]-release assays. Effector cells were plated into 96-well plates at various effector-to-target (E/T) cell ratios. Targets used were either peptide- or protein-pulsed BLCL or the human HER-2/neu–overexpressing tumor cell lines, SKOV3 and SKOV3-A2. Targets were labeled with 200 μCi ^{51}Cr for 1–2 hours at 37°C. BLCLs were labeled simultaneously with 10 μM peptide. Before mixing with effectors, the targets were washed two times with medium and resuspended to 1,000 targets/100 μl. The reaction was carried out for 4 hours at 37°C, after which the plates were centrifuged and 50 μl of medium from each well was assayed for [^{51}Cr] content in a scintillation counter. The percentage of specific activity was calculated using the following equation: percentage of specific lysis = (sample well release – basal release) / (detergent release – basal release).

Results

Patients with advanced-stage HER-2/neu–overexpressing cancers can be safely immunized with peptide-based vaccines. Nineteen subjects were enrolled on trial (Table 1). The median age was 52 years (range 36–55), and the median time from last chemotherapy was 10 months (range 1–75). Fourteen subjects received six vaccinations, two received four vaccinations, two received three vaccinations, and one received one vaccination. Postvaccination data are presented on 18 subjects who received more than one vaccine. At enrollment, 18 subjects had positive-recall antigen testing. The one subject who was anergic received one vaccine. This subject withdrew from the study because of worsening asthma. Toxicity was assessed using the National Cancer Institute Common Toxicity Criteria. Among all 19 subjects, there was one grade 2 skin rash characterized by a mild chronic urticaria that did not require treatment and resolved after completion of the vaccination series. Five subjects were followed a median of 12 months (range 7–17) after completing six vaccines.

Patients immunized with 15-aa HER-2/neu peptides develop HER-2/neu peptide– and protein–specific T-cell proliferative responses. T-cell proliferative responses were measured against 15-aa HER-2/neu peptides and the ECD and ICD proteins before, during, and after the vaccination series. As shown in Figure 1, before immunization proliferative responses were detected to p369–384 in 2 of 19 subjects (mean SI 1.0, range 0.6–2.4), to p688–703 in 2 of 19 subjects (mean SI 1.1, range 0.5–2.5), and to p971–984 in 1 of 19 subjects (mean SI 1.1, range 0.5–2.5).

After vaccination, 14 of 18 (83%) subjects had proliferative responses to at least one of the 15-aa HER-2/neu peptides contained within their vaccine formulations (Figure 2). After immunization, proliferative responses were detected to p369–384 in 14 of 18 subjects (mean SI 6.4, range 1.0–35.6), to p688–p703 in 7 of 18 subjects (mean SI 2.4, range 1.0–6.4), and to p971–984 in 10 of 19 subjects (mean SI 4.2, range 1.0–26.1) (Figure 1). The differences in means between the preimmunization responses and the maximal postimmunization responses were significant for all the peptides (p369–384, $P = 0.003$; p688–703, $P = 0.001$; p971–984, $P = 0.02$). Overall, new immunity was generated to p369–384, p688–703, and p971–984 helper peptides in 67%, 33%, and 50% of subjects, respectively (Figure 2). As a comparison, the mean maximal response to tetanus toxoid in the patient population was an SI of 14.8 (range 1.0–76.8) (Figure 1).

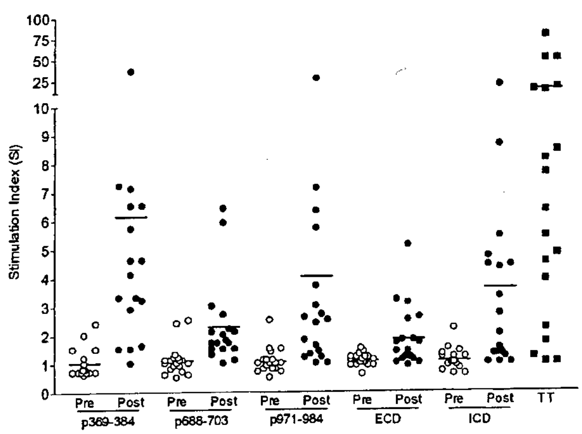


Figure 1
Patients immunized with a 15-aa HER-2/neu peptide–based vaccine develop HER-2/neu peptide-specific and protein-specific T-cell proliferation responses. Shown are the preimmunization (open circles) and maximal postimmunization (filled circles) proliferative responses (SI) for the HER-2/neu peptides, p369–384, p688–703, p971–984, and the HER-2/neu protein domains, ECD and ICD. For comparison, the maximal responses to tetanus toxoid (TT) are shown. Each symbol represents a measurement from a single unique subject, calculated from 24 replicates. The solid lines indicate the mean SI for the group.

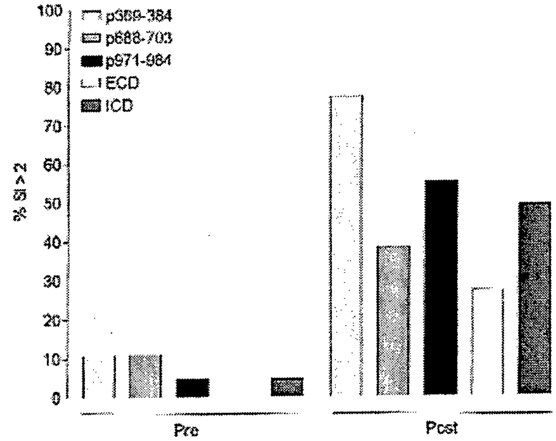
Table 1
Patient demographics

Diagnosis	Number of patients
Breast cancer	19
Stage III	4
Stage IV	14
Ovarian cancer	1
Stage III	1
Age, median years (range)	52 (36–55)
Time from last chemotherapy, median months (range)	10 (1–75)

Patients immunized with peptides also developed responses to the naturally processed and presented HER-2/neu protein. As shown in Figure 1, before immunization proliferative responses were detected to the ECD in 0 of 19 subjects (mean SI 1.1, range 0.6–1.5) and to the ICD in 1 of 19 (mean SI 1.1, range 0.6–2.2). After immunization, proliferative responses were detected to ECD in 5 of 18 subjects (mean SI 1.9, range 1.0–5.1) and to ICD in 9 of 18 subjects (mean SI 3.9, range 1–18). The differences in the mean postimmunization responses were significantly higher than the mean preimmunization SIs for both proteins (ECD, $P = 0.001$; ICD, $P = 0.004$). Overall, new immunity was generated to ECD protein in 28% and to ICD protein in 50% of subjects.

Patients immunized with 15-aa HER-2/neu peptides increase T-cell precursors to the HLA-A2 9-aa peptide epitopes contained within the longer peptide sequences of the vaccine peptides. Generation of HER-2/neu T-cell precursors to the 9-aa peptides, p369–377, p689–697, and p971–979, were evaluated in patients using an IFN-γ–based ELIspot (Figure 3). Figure 3a demonstrates that study patients had similar levels of viral-specific T-cell precursors compared with the levels of viral-specific precursors detected in a cohort of HLA-A2 volunteers. Before vaccination, IFN-γ–producing CD8 T-cell responses, defined as HER-2/neu peptide-specific precursors/10^6 PBMCs, were detectable to p369–377 in 2 of 15 (mean 12, range 0135), to p689–697 in 0/15 (mean 0, range 0-0) and to p971–979 in 3/15 (mean 21, range 0–217) subjects. As shown in Figure 3, after immunization CD8 T-cell responses were detected to p369–377 in 10 of 15 subjects (mean 75, range 0–471), to p688–703 in 5 of 15 subjects (mean 20, range 0–143), and to p971–984 in 12 of 15 subjects (mean 63, range 0–185). Overall, new CD8 T-cell immunity was generated to p369–377 in 62%, to p689–697 in 31%, and to p971–979 in 54% of subjects (Figure 4). The pre- and postimmunization responses to tetanus toxoid and the HLA-A2 peptides from influenza virus and CMV were not significantly changed ($P > 0.05$) as a result of vaccination with HER-2/neu peptides (data not shown).

Figure 2
The majority of patients could be immunized to HER-2/neu. Data are shown as the percentage of the population before immunization and after immunization that had a positive proliferation response (SI > 2) to each of the peptides in the vaccine, p369–384 (light gray bar), p688–703 (gray bar), p971–984 (filled bar), as well as to the HER-2 protein domains, ECD (open bar) and ICD (dark gray bar). The mean (range) preimmunization SI of patients considered as having a positive response to p369–384 was 2.4 (one patient only), to p688–703 was 2.5 (2.4–2.5), to p971–984 was 2.5 (one patient only), to ECD (no patients), and to ICD was 2.2 (one patient only). The mean (range) postimmunization SI of patients considered as having a positive response to p369–384 was 7.5 (2.9–35.6), to p688–703 was 3.5 (2.1–6.4), to p971–984 was 6.2 (2.4–26.1), to ECD was 3.3 (2.5–5.1), and to ICD was 6.2 (2.7–18).



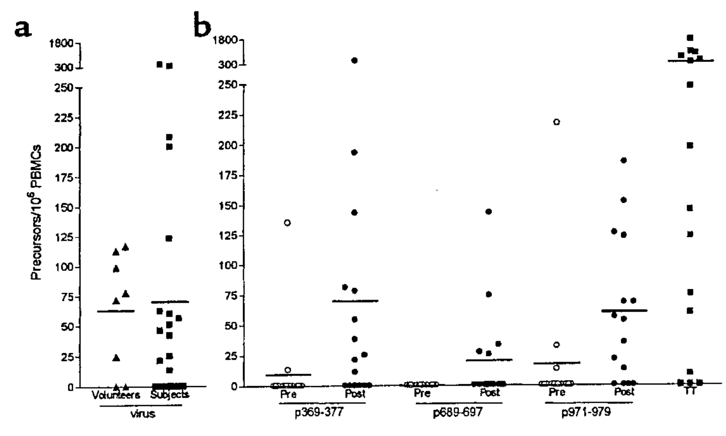
Peptide-specific T cells can lyse HLA-matched cells expressing HER-2/neu protein. Previous studies of peptide immunization indicate peptide-specific T cells may not have the capacity to lyse tumors (9, 27, 28). Thus, we assessed whether HER-2/neu peptide–specific T cells could lyse HLA-A2, HER-2/neu–expressing cell lines. As an example, Figure 5 demonstrates cytolytic activity against an HLA-A2 BLCLs transfected with HER-2/neu in a breast cancer patient after immunization. Postimmunization precursor frequencies in this representative subject after vaccination were 81, 27, and 56 precursors/10^6 PBMCs to p369–377, p689–697, and p971–979, respectively. The patient had no pre-existing peptide-specific T-cell precursors before immunization. A representative T-cell line established on this patient demonstrated a 40:1 E/T ratio, 22% lysis to p369–377, 35% lysis to p689–697, and 37% lysis to p971–979 (Figure 5). Furthermore, peptide-specific T cells were able to lyse HLA-A2$^+$ BLCLs expressing HER-2/neu protein (25% at 40:1 E/T). In an additional example, 21 CD8 peptide-specific T-cell clones were generated from an ovarian cancer patient, 0756, after vaccination (29). Shown in Figure 6 is the cytolytic activity of a representative peptide-specific clone against p369–377–loaded HLA-A2 BLCLs (25% at 40:1 E/T) or tumor cells expressing both HER-2/neu and HLA-A2 (18% at 40:1).

Patients immunized with 15-aa HER-2/neu peptides maintain HER-2/neu immune responses after immunizations have ended. To determine if CD8 T-cell responses were maintained after active immunization, five patients were followed between 7 and 17 months after the end of vaccination. All five of the patients maintained responses to two or more the HLA-A2 9-aa epitopes contained within their vaccine peptides (Table 2). At a median of 12 months after the last vaccination, the mean (n = 5 patients) precursor frequency, expressed as peptide-specific precursors/10^6 PBMCs, to p369–377 was 68 (range 38–118), to p689–697 was 22 (range 0–74), and to p971–979 was 43 (range 0–68).

Discussion

The cytolytic T-cell response is believed to be the critical immune effector arm in mediating potential antitumor immunity. Thus, studies of tumor immunity primarily have focused on the identification of MHC class I epitopes for tumor-associated antigens such as MAGE-1 (30), NY-ESO-1 (31), HER-2/neu (6, 7), tyrosinase (32), and gp-100 (33). In in vitro experiments, these epitopes, when presented in the context of MHC class I, activate CD8 T cells that can directly lyse tumors. Therefore, several clinical trials have been conducted assessing the feasibility and efficacy of cancer vaccination with peptide-

Figure 3
Patients immunized with a 15-aa HER-2/neu vaccine increase T-cell precursors to the encompassed HLA-A2 9-aa peptides. (**a**) Combined ELIspot responses (precursors/10^6 PBMCs) to HLA-A2–binding epitopes of influenza and CMV virus are shown. Data are from normal HLA-A2 volunteers (triangles) and study subjects (squares), with the mean delineated by a bar. (**b**) Preimmunization and maximal postimmunization ELIspot responses (precursors/10^6 PBMCs) to the HLA-A2 HER-2/neu peptides, p369–377, p689–697, p971–979, in subjects are shown. For comparison, the maximal responses to TT are shown. Each symbol represents a measurement from a single unique subject, calculated on six replicates.



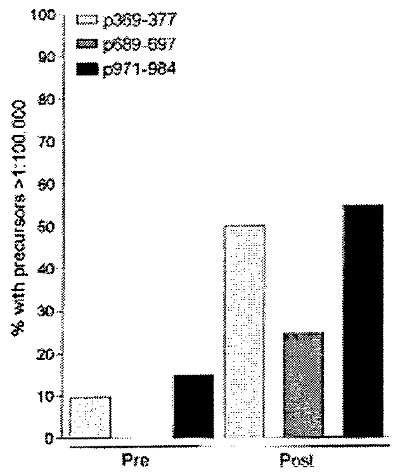


Figure 4
The majority of patients could be immunized to HLA-A2 HER-2/neu peptides. Data are shown as the percentage of the population before immunization and after immunization that had a detectable ELIspot response to each of the HER-2/neu, HLA-A2 peptides, p369–377 (light gray bar), p689–697 (dark gray bar), p971–979 (filled bar). The mean (range) preimmunization HER-2/neu–specific precursor frequency of patients considered as having a positive response to p369–377 was 125 (32–217, two patients only), to p689–697 (no patients), and to p971–979 was 74 (13–135, two patients only). The mean (range) maximal postimmunization HER-2/neu–specific precursor frequency of patients considered as having a positive response to p369–377 was 111 (11–417), to p689–697 was 60 (25–143), and to p971–979 was 97 (13–185).

based MHC class I peptides to generate tumor-specific CD8 T cells. Several problems have been identified with MHC class I peptide vaccination, including the inability to generate (a) peptide-specific precursors that directly recognize naturally processed antigen, (b) a significant precursor frequency, and (c) long-lasting immunity.

The requirement of CD4 help to initiate and sustain a CD8 response is well established and has led to the development of antitumor vaccines that attempt to induce both T-cell subsets (1, 34). In the absence of defined tumor-antigen MHC class II epitopes needed to activate CD4 T cells, immunization strategies have been employed combining tumor antigen MHC class I epitopes with universally recognized MHC class II epitopes such as PADRE (35) and the promiscuous epitopes of tetanus toxoid (36). Although immunity to the helper epitopes is usually robust, responses to the antigen of interest have been limited. For example, Brander and colleagues reported that inclusion of the promiscuous tetanus epitope, p30, into a vaccine formulation containing an HIV HLA-A2 peptide epitope did not result in immunization to the HIV epitope but significantly reactivated the memory response to the tetanus peptide (37). Similarly, PADRE was unable to significantly induce immunity to two human papilloma virus–derived, HLA-A2 peptides in a phase I clinical trial (16). In contrast to these strategies, providing CD4 help within the same antigenic background has been successfully used to boost CD8 responses (12, 17). In fact, immunizing with an MHC class II epitope that encompasses an MHC class I-binding motif within its natural sequence resulted in effective immunity in a murine lymphocyte choriomeningitis virus model (17). Previous investigations by our group identified putative T-helper epitopes of the HER-2/neu protein that contained HLA-A2 motifs (2). By providing HER-2/neu-specific MHC class II and MHC class I epitopes simultaneously, we hoped to overcome the problems associated by immunizing with MHC class I epitopes alone.

The peptide-specific T cells that were generated in vivo in the present study were able to lyse tumor cells. The inability of peptide-specific T cells generated by vaccination to directly recognize naturally processed and presented antigen has been reported for some MHC class I epitopes, including those derived from HER-2/neu (9)

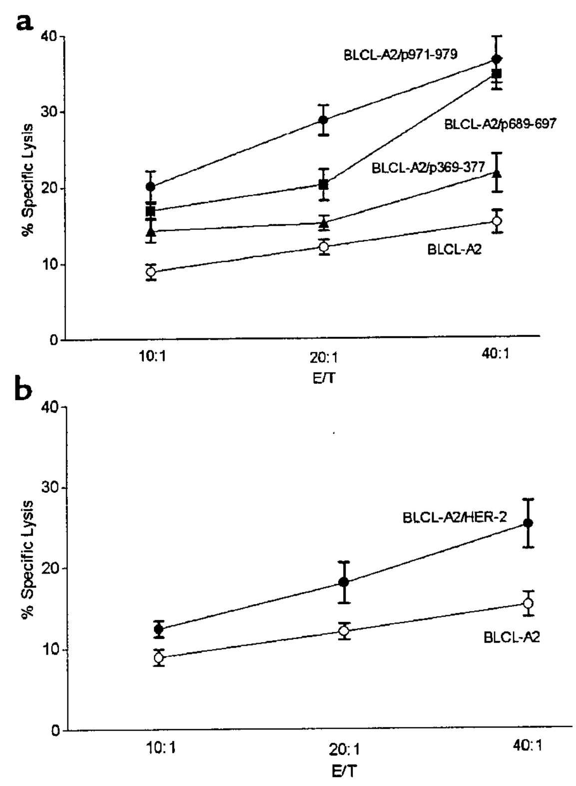


Figure 5
Peptide-specific T cells isolated from a breast cancer patient after immunization can lyse HLA-A2 cells overexpressing HER-2/neu protein. PBMCs from a representative patient, 0107, were examined for cytolytic activity against BLCL-A2 alone (open circles), peptide-loaded A2-BLCLs (filled symbols), or HER-2/neu–expressing BLCL-A2 (open squares) at three different E/T ratios. The peptides (p369–377, p689–697, p971–979) used to pulse the BLCL-A2 were the HLA-A2–binding peptides encompassed in the 15-aa HER-2/neu vaccine. Each point represents the mean of three replicates.

Table 2
CD8 T-cell responses are maintained after vaccination

Subject	p369–377		p689–697		p971–979	
	Maximal response	Latest response[A]	Maximal response	Latest response[A]	Maximal response	Latest response[A]
8302	1:12,800	1:21,300	1:30,300	1:91,000	1:25,000	1:25,000
4716	1:8,500	1:8,500	<1:100,000	<1:100,000	1:18,700	1:20,000
4723	1:5,200	1:18,500	1:7,000	<1:100,000	1:14,700	1:14,700
2859	1:26,300	1:26,300	1:13,500	1:13,500	1:76,900	<1:100,000
0107	1:12,300	1:12,300	1:37,000	1:37,000	1:17,900	1:17,900

Data are presented as ratio of antigen-specific T cells to PBMCs. [A]Response measured between 7 and 17 months (median = 12) after final vaccination.

and MART-1 (28). Peptide-specific T cells that do not respond to endogenous antigen may be detected after immunization if target cells (e.g., tumor cells) do not naturally process or present the epitope in sufficient quantities to stimulate recognition. Alternatively, immunization with excessive quantities of class I–restricted peptide may result in the generation of peptide-specific T cells that are of low affinity and would not be activated by the level of naturally occurring peptide in tumor cells. Vaccinating with longer peptides encompassing class I motifs, as in the present study, may allow processing of the peptide and presentation in class I MHC at levels that more closely mimic those present on tumors.

The majority of patients in the current study generated viral-like levels of HER-2/neu–specific CD8 T-cell precursors. Immunization with MHC class I cancer vaccines often has resulted in undetectable or low-level immune responses. For example, Pass and colleagues reported that only two of seven (29%) melanoma patients vaccinated with the gp100 HLA-A2–binding motif, g208, developed detectable peptide-specific precursors (8). In a parallel study, they also observed no detectable response in another cohort vaccinated against the HLA-A2 MART-1_{27-35} epitope (8). Our success at generating high levels of peptide-specific precursors to HER-2/neu HLA-A2 peptides was most likely due to the patient population selected, having either low-level or nondetectable disease and an excellent performance status. Scheibenbogen and colleagues have demonstrated that the presence of antigen-specific immune reactivity in melanoma patients can be correlated with disease being in remission (38). Our goal, like that in infectious disease, is to develop vaccination strategies that prevent disease rather then treat disease. The inability of vaccines to eradicate actively growing tumors has been clearly shown in animal models (39). In our study 86% of patients generated increased frequencies of HER-2/neu–specific CD8 T cells. The mean HER-2/neu (all peptides) precursor frequency of $49/10^6$ PBMCs was very similar and within one SD to the mean viral (flu and CMV) precursor frequency of $65/10^6$ PBMCs. Furthermore, the levels of viral precursors measured in the present study are consistent with those observed by Scheibenbogen in a cohort of melanoma and noncancer-bearing patients (38). These findings together raise the important question as to whether levels of cancer immunity that are similar to viral immunity would be sufficient to protect against cancer relapse. This question can be answered only in the context of clinical studies designed to correlate level of immunity generated after immunization with protection from cancer relapse or development of disease.

Our vaccination strategy also resulted in persistent peptide-specific T-cell precursors. Over the past decade, immunization with CD8 T cell–inducing epitopes was associated with only short-lived responses (40). However, strategies are being developed to lengthen the duration of the response. It is likely that our strategy resulted in long-lived CD8 T-cell responses due to the concurrent activation of CD4 T cells. Both murine antiviral and antitumor models have clearly established the important role of CD4 T cells in maintaining a persistent CD8 T cell response (14, 34, 41). Using a different strategy, Stewart and Rosenberg have found that gp100-specific CD8 T-cell responses are long-lived in melanoma patients immunized with a modified gp100 MHC class I–binding peptide (42).

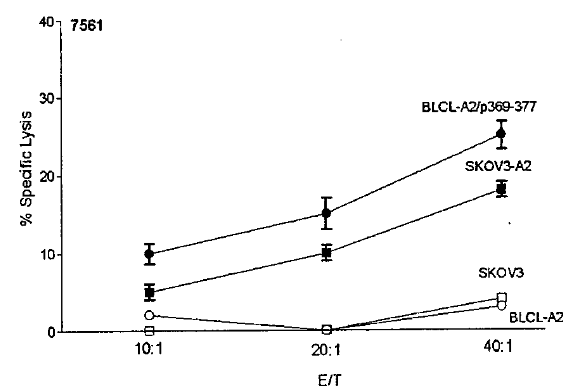


Figure 6
Peptide-specific T-cell clones isolated from an ovarian cancer patient after immunization can lyse HLA-A2+ tumor cells overexpressing HER-2/neu protein. A p369–377–specific clone was examined for cytolytic activity against BLCL-A2 alone (open circles), p369–377-loaded BLCL-A2 (filled circles), or the HER-2/neu–overexpressing tumor cells, SKOV3 (open squares) and SKOV3-A2 (filled squares). SKOV3-A2 are SKOV3 cells stably expressing HLA-A2. Each point represents the mean of three replicates (± SEM). The absence of errors indicates a standard of the mean less than 1%.

In summary, the primary use of cancer vaccines is most likely to prevent, rather than eradicate, malignancy. In this study immunization of cancer patients against HER-2/neu was demonstrated with a peptide-based vaccine consisting of helper T-cell epitopes, each containing an HLA-A2 motif. Active immunization resulted in the generation of both CD4 and CD8 T-cell immunity. The resulting peptide-specific T-cell precursors recognized naturally processed HER-2/neu protein and the immunity was long-lived.

Acknowledgments

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IL-12 Enhances *In Vitro* Expansion and Function of Peptide-Specific T Lymphocytes

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Short Title: IL-12 activates peptide-specific T lymphocytes

Keywords: IL-12, T lymphocyte, cytokine, influenza, peptide.

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Abstract

Growth of antigen-specific T cells *in vitro* is critical for understanding their potential for use therapeutically. The use of IL-2 has become the mainstay for extended maintenance of antigen-specific T cell lines and clones *in vitro*. However, other cytokines, such as IL-12, have been recently described and can have important additive effects on T cell growth when used with IL-2. In this study, the effects of IL-12, along with IL-2, on the generation of peptide-specific T cells lines were evaluated. Studies were performed in volunteer donors expanding influenza-specific, MHC class I-restricted T cells *ex vivo*. IL-12 was added, along with IL-2, to T cell cultures stimulated with an MHC class I-restricted, 9-mer peptide derived from influenza matrix. The addition of IL-12 resulted in increased cell yield compared with IL-2 alone. IL-12 resulted in increased CD8/CD4 ratios and peptide-specific IFN-γ-secreting precursors. Subsequent studies evaluated the role of IL-12 in expanding T cells specific for an MHC class II-restricted (helper) epitope of HER-2/neu, a tumor antigen. IL-12 enhanced both cell number and function of HER-2/neu peptide-specific T cells expanded *in vitro* compared to IL-2 alone. These findings demonstrate that the addition of IL-12 to IL-2 can effectively increase cell yield, alter cell population phenotype, and enhance production of type I cytokines from *in vitro*-expanded T cells stimulated with either MHC class I- or MHC class II-restricted viral or tumor peptide epitopes.

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Abbreviations

IVS, *in vitro* stimulation; PBST, phosphate-buffered saline containing Tween-20; ELIspot, enzyme-linked immunosorbent spot assay; HLA-A2, Human Leukocyte Antigen A2.

Introduction

The growth of human antigen-specific T cell lines and clones *in vitro* is important for understanding basic T cell biology and the potential for the use of the T cells in therapeutic applications such as adoptive T cell therapy. In some cases however, currently used *ex vivo* culture conditions may be inadequate for expansion of T cells that retain antigen specificity. An important variable of *in vitro* culture that can be manipulated to maintain or enhance antigen-specific function during *ex vivo* expansion is the cytokine environment. An experimental goal in infectious disease and tumor immunology is to define *in vitro* cytokine conditions that promote propagation of all T cell subsets (e.g. CD4+ and CD8+) while preserving or enhancing antigen-specific function during culture of human T cells with peptide antigen.

The discovery and characterization of multiple cytokines that stimulate T cell activity such as IL-2, IL-7, and IL-12, has the potential to greatly facilitate *in vitro* expansion techniques. One cytokine that has received considerable attention is IL-12, originally known as natural killer cell stimulatory factor and cytotoxic lymphocyte maturation factor [1-3]. IL-12 is a heterodimeric cytokine, produced by B cells, macrophages, and professional antigen-presenting cells.

The effects of IL-12 on the *in vitro* culture of T lymphocytes is best understood in rodents. A recent study indicates that in addition to peptide antigen and costimulation, murine T cells need an additional signal in order to proliferate and develop robust effector functions in an antigen-specific manner. IL-12 is capable of providing that additional signal for CD8, but not CD4, peptide-specific murine T lymphocytes [4]. In humans, IL-12 also has been reported to have multiple effects on T cell function when added together with low-dose IL-2 [1,2,5-7]. For example, Gerosa et al., have shown that IL-12, added during the first few days of cloning CD4+

and CD8+ human T cell clones of undefined antigen specificity, resulted in priming for high production of IFN-γ [8]. In addition, the cytotoxic activities of HIV-1-specific human CTL lines were augmented by inclusion of IL-12 into the cell culture medium [3,7]. Although, these results support the use of IL-12, in addition to IL-2, for *in vitro* culture of human T cells, it still is unclear what the effects of the cytokine are on enhancing non-cytolytic functional attributes such as increased antigen-specific cytokine production. Furthermore, it also remains to be determined if IL-12 can be the additional signal for maximizing responses of antigen-specific CD4 T cells.

In the present study, the *in vitro* effects of IL-12, along with IL-2, on human T cell line growth, phenotype, and antigen-specific function were evaluated using either MHC class I- or MHC class II-restricted peptide antigens. In our efforts to define the optimal T cell growth conditions, we used peptide antigens to eliminate the variability that may be observed by the use of whole protein antigens which, generate multiple epitopes and may confound characterizing the effects of IL-12.

The MHC class I peptide used was the well-defined HLA-A2 restricted 9-mer peptide derived from the influenza virus matrix protein [9,10]. Detectable levels of CD8+ precursors specific for this 9-mer have been observed in normal HLA-A2+ volunteer donors by both tetramer analysis and cytolytic T cell assays [11]. Furthermore, this peptide has been routinely used as a standard in many immunological investigations [12-14]. In the present study, the influenza matrix peptide was used for examining the effects of IL-12 on the expansion of peptide-specific CD8+ T cells. A previously defined epitope, p776-790, of the tumor antigen HER-2/neu, was used to evaluate *ex vivo* expansion of MHC class II restricted T cells [15,16]. HER-2/neu is a well-defined tumor-associated protein to which we have been able to generate immunity using peptide vaccination and is a model in our laboratory for assessing immune responses to tumor antigens [17]. IL-12, supplementing IL-2, was evaluated for effect on the expansion, phenotype, and function of antigen-specific human T cells.

Methods

Materials: All peptides were manufactured by United Biochemical Inc. (Seattle, WA) and were greater than 95% pure as assessed by HPLC and mass-spectrometric analysis. Peptides used in this study were HLA-A2 binding flu-matrix peptide, GILGFVFTL (pFlu) [9]; HLA-A2-binding EBV peptide, YLLEMLWRL [18]; and HER-2/neu peptides p42-56 (p42-15), HLDMLRHLYQGCQVV [17] and p776-790 (p776-15), GVGSPYVSRLLGICL [15]. Sterile nitrocellulose-backed microfiltration 96-well plates were purchased from Millipore Corp (Bedford, MA). Anti-IFN-γ monoclonal antibody was purchased from R&D Systems (Minneapolis, MN). Streptavidin-alkaline phosphatase and AP-colorimetric reagents were from BioRad (Hercules, CA). IL-12 (batch # 4D18I002, 1.7×10^7 U/mg) was a generous gift from the Genetics Institute (Cambridge, MA). Anti-CD8, anti-CD4, anti-αβ-TCR, anti-CD33, anti-CD56, biotinylated anti-IFN-γ, biotinylated anti-TNF-α antibodies and purified recombinant human TNF-α were obtained from Pharmingen (San Diego, CA), ultravidin-phycoerythrin from Leinco Technologies (Ballwin, MO), human AB^+ serum from Valley Biomedical, Incorporated (Winchester, VA), and Immulon IV ELISA plates from Dynex Technologies (Chantilly, VA). TMB peroxidase substrate was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

Patient and Volunteer Donors. PBMC were obtained from volunteer donors by leukapheresis after informed consent. Breast cancer patients participating in a Phase I trial of HER-2/neu peptide vaccines [17] were leukapheresed at the completion of the trial after informed consent.

PBMC derived from those leukaphereses were used in these studies. HLA typing of both donors and patients was performed by Puget Sound Blood Bank (Seattle, WA).

Preparation of PBMC from leukapheresis products: PBMC were isolated by density gradient centrifugation as previously described [17]. Cells were aliquoted and cryopreserved in liquid nitrogen in freezing media (90% fetal bovine serum and 10% dimethylsulfoxide) at a cell density of 25-50 x 10^6 cells/ml.

In vitro stimulation (IVS) of PBMC with peptide: In all experiments, except as indicated, PBMC were stimulated *in vitro* for 12 days in the presence of either 9-mer or 15-mer peptide. For each experiment, 40-80 x 10^6 cryopreserved cells were rapidly thawed, washed 2 times in 10 mls of RPMI-1640 containing streptomycin, penicillin, and 10% human AB serum (complete medium), and resuspended at 1.0 X 10^6 cells/ml in the same medium and incubated in a humidified incubator at 37°C at 5% CO_2. Peptide was added on day 0 to a concentration of 10 μg/ml. Ten U/ml of IL-2 and 1-100 ng/ml of IL-12 were added on days 4 and 8 and the cells were further incubated for 4 days.

T-lymphocyte proliferation: Thymidine incorporation was measured as previously described [17,19]. Briefly, on day 0, purified PBMC were plated at 2.5 x 10^5 cells per well, in 200 μl of complete medium in the absence or presence of 50 μg/ml of various antigens. Assays were performed in 24-well replicates for each antigen. On day 5, ^{3}H-thymidine was added for 8 hours. The plates were then harvested onto filtermats and the incorporated isotope was measured in a

Wallac Trilux scintillation counter. Data is expressed as a stimulation index (S.I.), defined as the mean value of 24 experimental wells/mean value of 24 control wells.

Flow cytometry: Donor or patient PBMC were carried through one IVS and washed in FACS staining buffer (PBS containing 20 mM glucose and 0.5% BSA). Cells were stained in 50 μl of FACS staining buffer containing control antibody, anti-CD4, anti-CD8, anti-αβ-TCR, anti-CD3, or anti-CD56 for 1-2 hr at 4°C. Following 2 washes the cells were fixed in PBS containing 1% paraformaldehyde and analyzed by flow cytometry. Data presentation and statistical analysis were completed using CellQuest flow cytometry software (BD Immunocytometry Systems, San Jose, CA).

Cytokine secretion: Following IVS, cells were harvested, counted, washed and resuspended in replicate at 200 μl/well in 96-well plates at 1.25×10^6 cells/ml in complete media. Ten μg/ml peptide and irradiated, autologous PBMC at 2.5×10^5/well were added and the cells incubated for a further 40 hours at 37°C. One hundred microliters of media were removed from each well and evaluated for cytokine content by ELISA. Immulon IV ELISA plates were coated overnight at 4^0C with 50 μl of mouse anti-TNF-α monoclonal antibody at 2.5 μg/ml in carbonate buffer (15 mM Na_2CO_3, 35 mM NaH/CO_3, pH 8.0). Wells were blocked 2 hours at room temperature with 200 μl of PBS containing 1% BSA and 0.5% Tween-20. The plates were washed 4 times using PBS containing 0.1% Tween-20 (PBST). Cell culture supernatants were added at 100 μl/well and the plates were further incubated overnight at 4^0C. The plates were washed 4 times with PBST. One hundred microliters PBST containing 10% NGS and 0.5 μg/ml biotinylated anti-

TNF-α antibody were added to wells and the plates were further incubated for 2 hours at room temperature followed by washing 4 times with PBST. The plates were subsequently incubated with 100 μl/well of PBS containing 1:3000 streptavidin-horseradish peroxidase, washed, and developed with 100 μl/well TMB peroxidase substrate. The developing reaction was terminated with 1N hydrochloric acid and the OD was measured at 450 nm optical wavelength using a Molecular Devices kinetic microplate reader. Purified TNF-α was analyzed in parallel and the OD values of the samples were used to establish a standard curve (ng/ml). Statistical analysis was performed using the Student's t test (Microsoft Excel 97).

Enzyme-linked immunosorbent spot (ELIspot): A 3-day ELIspot assay was used to determine precursor frequencies of peptide-specific CD8+ T lymphocytes as previously described [20]. Briefly, on day 1, cultured PBMCs were plated into 96-well plates in 6-well replicates in 200 μl complete medium in the presence or absence of 10 μg/ml antigen. Irradiated, autologous PBMC were added at a 1:1 ratio. On day 1, nitrocellulose-backed 96-well plates (NC-plate) were coated with anti-IFN-γ antibody at 50 μl/well using a concentration of 4 μg/ml in PBS. On day 2 the NC-plate was washed 3 times with PBS and blocked for 2 hours with PBS containing 2% bovine serum albumin followed by 3 washes with PBS. On day 2, 190 μl of media were carefully removed from each well of the culture plate and the cells were gently resuspended in 200 μl of media. The cells were transferred into the NC-plate in a volume of 200 μl/well. The NC-plate was incubated at 37°C for a further 20 hours followed by washing 6 times using PBS containing 0.05% Tween-20. The plate was incubated for 2.5 hours at room temperature in biotinylated anti-IFN-γ antibody at 100 μl/well using a concentration of 2.5 μg/ml. The plate was washed 6 times with PBS and further incubated with streptavidin-alkaline phosphatase (1:1000 in PBS, 100 µl/well) for 2 hours at room temperature. The plate was washed 6 times in PBS and incubated in 100 µl/well AP-colorimetric substrate for 20-30 minutes, rinsed with cool tap water and allowed to completely dry. The spots were then enumerated using a dissecting microscope. Precursor frequencies were calculated by subtracting the mean number of spots obtained from the no antigen control wells from the mean number obtained in the experimental wells. Statistical analysis was performed using the Student's t test (Microsoft Excel 97).

Results

The addition of IL-12 to IL-2 increases total cell yield after IVS with flu-matrix peptide using PBMC derived from volunteer donors. The effects of IL-12 on cell growth, in terms of total cell yield, were assessed using PBMC from HLA-A2^{+} volunteer donors expanded on pFlu. The HLA-A2 flu-matrix peptide-specific precursor-frequencies, determined by ELIspot at the time of initial evaluation for each donor were D1, 1:9,000; D2, 1:80,000; and D3, <1:100,000. PBMC derived from each of these donors were incubated through one IVS using flu-matrix 9-mer in the absence or presence of IL-12 (1, 10, or 100 ng/ml) and cell yields were assessed by counting viable cells using trypan blue staining. Overall, IL-12 induced an increase in cell number compared to cultures carried through one IVS in the presence of IL-2 alone (Fig. 1A, $p=0.04$), although one donor, D3 did not demonstrate an increase in total cell number. Panel B shows cell yields increased using a variety of IL-12 doses, compared to IL-2 alone ($p=0.02$ at 10 ng/ml and $p=0.04$ at 100 ng/ml) although there was no difference in magnitude of expansion with the various concentrations of IL-12 (1, 10, 100 ng/ml IL-12, Fig. 1B, $p>0.05$). Considerable membrane blebbing of lymphocytes was often observed by light microscopy at 100 ng/ml, potentially indicating toxicity as a result of high IL-12 exposure. Thus, the remaining data are presented at concentrations ranging between 1 and 10 ng/ml. The effects of IL-12 on cell growth were dependent on the presence of IL-2 (data not shown).

The addition of IL-12 to IL-2 can increase the CD8/CD4 ratio of T cells after IVS with flu-matrix peptide. In order to assess the effects of IL-12 on T cell subsets, PBMC from the same 3 volunteer donors shown in Fig. 1 were stimulated *in vitro* using the flu-matrix 9-mer in the presence or absence of IL-12. The cultures were then examined by flow cytometry in the lymphocyte gate for cell surface expression of CD8, CD4, CD3, αβ-TCR, or CD56. Shown in Fig. 2 are the CD8/CD4 ratios obtained from the three normal donors described in Fig. 1 following incubation in IL-2 alone or IL-2 with either 1 or 10 ng/ml IL-12. IL-12 at all concentrations tested was able to significantly enhance the CD8/CD4 ratios in all experiments. All observed increases in the CD8/CD4 ratios were significantly different ($p<0.05$) than that observed with IL-2 alone. The average percentage of cells expressing CD3 (79±10, IL-2 vs. 87±6, IL-2/IL-12), αβ-TCR (87±5, IL-2 vs. 90±3, IL-2/IL-12), or CD56 (9±4, IL-2 vs. 8±2, IL-2/IL-12) was not changed by the inclusion of IL-12. In order to determine if the effect of IL-12 on increasing the CD8/CD4 ratios required the presence of antigen, cultures of volunteer donor D1 PBMC were incubated in the absence or presence of the flu-matrix peptide. The addition of IL-12 in the absence of antigen resulted in an increase in CD8/CD4 ratio from 0.1 to 0.3 (Fig. 3). The addition of antigen in the absence of IL-12 increased CD8/CD4 ratio from 0.1 to 1.0. The inclusion of both IL-12 and antigen increased the CD8/CD4 ratio from 0.1 to 2.0. Similar results were obtained with the other donors. Thus, the addition of antigen markedly increased in the CD8/CD4 ratio and was synergistic with IL-12.

The addition of IL-12 to IL-2 increases flu-matrix peptide-specific IFN-γ secreting precursors from PBMC after IVS with flu-matrix peptide. ELIspot analysis was used to determine if IL-12 was able to induce increased flu-matrix peptide-specific IFN-γ production by CD8+ T cells. As shown in Figure 4, panel A, the addition IL-12 to cultures containing 10 U/ml IL-2 and 10 μg/ml flu-matrix peptide resulted in significantly elevated flu-matrix-specific IFN-γ-secretion compared to control cells receiving IL-2 alone ($p<0.001$, D1; $p=0.01$, D2; $p=0.002$, D3). In contrast, when the same flu-matrix peptide-cultured cells were tested against an HLA-A2 binding EBV peptide [18], no significant increases in precursors specific for the non-stimulating peptide were observed in response to IL-12 (panel B, $p>0.05$).

The addition of IL-12 to IL-2 increases total cell yield after IVS with HER-2/neu helper peptide using PBMC derived from cancer patients. Patients 1276, 6622, and 0982 were vaccinated with a HER-2/neu peptide-based vaccine consisting of peptides, 15 amino acids in length, which have been defined as helper epitopes [17]. The patients had no detectable preexisting immunologic response to the immunizing peptides. However, after immunizations were complete, all 3 patients had detectable antigen-specific proliferation responses (Fig. 5). PBMC derived from each patient donor were used to assess the effects of IL-12 on antigen-specific cell growth, similar to that described for flu matrix in the volunteer donors. As shown in Figure 6, the addition of IL-12 to IL-2 resulted in increased cell yield following 1 IVS with HER-2/neu helper peptide, p776-790, one of the immunizing peptides in each patient's vaccine.

The addition of IL-12 to IL-2 had no effect on the CD8/CD4 ratios after IVS with HER-2/neu helper peptide using PBMC derived from cancer patients. An analysis of cell surface marker expression, similar to that using normal donor PBMC, was also carried out using cancer patient PBMC carried through one IVS with HER-2/neu helper peptide, p776-790, in the absence or presence of IL-12 (Fig. 7). The cultures were examined by flow cytometry in the lymphocyte gate for cell surface expression of CD8, CD4, CD3, and $\alpha\beta$-TCR. The mean number of cells expressing CD3 and $\alpha\beta$-TCR in the gate was $82 \pm 6\%$ and $84 \pm 5\%$, respectively. A minimal increase in CD8/CD4 ratio was only detected using PBMC from patient donor 6622. PBMC from patient donors 1276 and 0982 demonstrated a slight decrease in the CD8/CD4 ratio.

The addition of IL-12 to IL-2 increases peptide-specific TNF-α release by T cells after IVS with HER-2/neu helper peptide using PBMC derived from cancer patients. The effects of IL-12 on enhancing tumor antigen peptide-specific function were assessed using TNF-α secretion assays. TNF-α was used, as an alternative to IFN-γ, to assure that IL-12 was mediating a type I cytokine (i.e. Th1) response. As shown in Fig. 8, IL-12 was able to enhance tumor antigen-induced TNF-α release compared to cultures incubated in IL-2 alone, in PBMC from all three patient donors. In all cases, the release of TNF-α induced by peptide antigen in cultures of T cells incubated with IL-2 and IL-12, was significantly greater than that induced by irrelevant antigen or control medium ($p>0.05$).

Discussion

In vitro generation of human T cell lines and clones is widely used to study many aspects of T cell biology including TCR clonality and validation and specificity of T cell antigen-recognition. In addition, in tumor systems T cells are expanded *in vitro* for the purpose of identifying immunological responses following vaccination and potentially for therapeutic infusion in patients with cancer. However, the current methodologies for growing human T cells are not always adequate and, in many cases, fail to expand appropriate antigen-specific T cell subsets.

The purpose of the studies presented here was to develop *in vitro* expansion conditions that promote propagation of all T cell subsets (e.g. CD4+ and CD8+) while preserving or enhancing antigen-specific function during culture of human T cells with peptides. Cytokines represent one component of growth medium that can be readily manipulated. Evaluating the role of cytokines during *in vitro* growth can be confounded by many factors related to activation of T cells. These include the presence or lack of other cytokines (e.g. IL-2) and the type of antigen present (e.g. whole protein or peptide). For example, the addition of whole protein antigen will result in the processing and presentation of multiple epitopes. T cells recognizing some of these epitopes may not be respond to certain cytokines due to subset differences (i.e. Th1 vs Th2). For example, Th2 T cells do not possess the IL-12 receptor and thus would not respond to the addition of exogenous IL-12 [21,22]. *In vitro* expansion using whole proteins could result in the outgrowth of a predominantly Th2 population which would not adequately reflect the effects of the cytokine. The use of MHC class I- or class II-restricted synthetic peptides during culturing eliminates many of these variables, such as generating a polyclonal response and effects resulting from impurities in the protein preparation. Indeed, studying the effects of cytokines in a oligoclonal or monoclonal setting would be ideal. In the present study, the *in vitro* effects of IL-12, on bulk-cultured PBMC, were examined using exogenous peptide antigen along with low dose IL-2 (10 U/ml). We initially employed a MHC class I-restricted, CTL epitope of flu-matrix and the findings were extended to a model employing a MHC class II-restricted helper epitope of HER-2/neu, a defined tumor antigen. The findings presented demonstrate that the addition of IL-12 to IL-2 can (1) increase total cell yield (mostly CD3+/αβTCR+ T cells), (2) in some cases, affect the CD8/CD4 ratio, and (3) augment numbers or function of cells producing type I cytokines. Furthermore, these novel findings demonstrate that IL-12 provides an important additional signal for enhanced proliferation and function of both human CD8 and CD4 T cells expanded *ex vivo* in the presence of foreign or self peptide antigen.

The observation that IL-12, when added along with IL-2 to T cells in cultures, will lead to increased cell yield following *in vitro* expansion with an MHC class I-restricted 9-mer epitope, is consistent with earlier findings that IL-12 increases the proliferation rate of the CD8+ subset of T lymphocytes [1,4]. However, we observed that the effects on cell growth were not dose-dependent over the concentration range of 1-100 ng /ml (10-1000 U/ml). Higher doses caused membrane blebbing in cultured lymphocytes. In addition, in the flu matrix HLA-A2 peptide system, IL-12 also consistently increased the CD8/CD4 ratio. The change in CD8/CD4 ratios was not dependent on, but was significantly enhanced by the inclusion of peptide into the culture media. Similar findings were reported in a study characterizing the role of IL-12 in an *in vitro* HIV-1 antigen model [23]. In those experiments, IL-12 significantly elevated the CD8/CD4 when added along with whole protein antigen. The present study extends these observations to peptide antigens.

It is thought that anti-tumor responses are cell-mediated and regulated by Th1 CD4+ T cells that produce type I cytokines such as IL-2, IFN-γ, and TNF-α. Type I cytokines create a tumor environment able to support priming and clonal expansion of CD8+ T cells specific for tumor antigens. We questioned whether IL-12 would be effective at enhancing Th1 responses in the HER-2/neu model. In those experiments, we used the HER-2/neu derived peptide p776-790 [15,16].

Similar to the flu-matrix 9-mer-peptide model, IL-12 along with IL-2 promoted increased cell yield in cells cultured with the HER-2/neu helper epitope, p776-790. Unlike the flu-matrix 9-mer model, however, the CD8/CD4 ratio was not altered to the same extent in response to IL-12 stimulation. This observation is most likely directly due to the peptides used since an MHC class I restricted peptide, like the flu-matrix 9-mer, favors activation and expansion of CD8+ T cells. In contrast, longer "helper" epitopes, like p776-790, are associated predominantly with the activation of CD4+ T cells. Furthermore, IL-12 along with IL-2 was able to significantly enhance TNF-α production specifically for the IVS peptide, p776-790. These results demonstrate that IL-12 can create an environment capable of sustaining Th1 responses.

Our findings corroborate recent hypotheses in rodent models that maximal stimulation of T cell proliferation and function requires a third "danger" signal other than antigen and co-stimulation [24,25]. IL-12 has been identified as a potential third signal for CD8 T cells and IL-1 for CD4 T cells [4]. In that study, Curtsinger and colleagues have shown that IL-12 enhances proliferation and cytolytic activity of purified ova-specific mouse T cells. Proliferation of CD4 T cells in contrast was not increased by IL-12 but rather IL-1. In our studies, in the human T cell system, we observed that IL-12 acts on both peptide-specific CD8 and CD4 T cells by increasing proliferation or cytokine release. Furthermore, these effects were specific for the peptide used during *in vitro* stimulation and not an irrelevant peptide. Thus, IL-12 may represent an important third signal for the both human CD4 and CD8 T cells.

In summary, the results presented here demonstrate that IL-12, when added to *in vitro* cell cultures during IVS with IL-2, and either MHC class I- or MHC class II-restricted peptide epitopes, enhances peptide-specific cell growth, alters the phenotype of the cell population, and enhances peptide-specific type I cytokine production. Thus, *in vitro*, IL-12 is able to potentiate a type 1 cytokine response to peptide antigen.

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Figure Legends

Figure 1: The addition of IL-12 to IL-2 increases total cell yield after IVS with flu-matrix peptide using PBMC derived from volunteer donors. Panel A: Data is shown as the average (± SEM) and individual increase in cell numbers for all 3 volunteer donors, D1, D2, and D3 from cultures stimulated with 10 μg/ml flu-matrix peptide with 10 U/ml IL-2 alone (open bars) or with 10 ng/ml IL-12 and 10 U/ml IL-2 (filled bars) as described in Materials and Methods. Panel B shows the mean (± SEM) cell yield for donors D1, D2, and D3 for IL-2 alone or for each dose of IL-12 tested. The values at each dose of IL-12 were compared with the values for IL-2 alone using the Student's t test. Results shown are from 3 independent experiments, each using PBMC from different donors, D1, D2, D3.

Figure 2: The addition of IL-12 to IL-2 increases the CD8/CD4 ratio of T cells after IVS with flu matrix-peptide. PBMC from volunteer donors (D1, D2, D3) were cultured as described in Fig. 1, and analyzed for phenotype by flow cytometry. Data shown are obtained from a minimum of 5000 gated events. Shown are the CD8/CD4 ratios of cultures stimulated with antigen and IL-2 alone (open bars), IL-2 + 1 ng/ml IL-12 (gray bars), or IL-2 + 10 ng/ml IL-12 (black bars).

Figure 3: IL-12-induced increase in CD8/CD4 ratios is enhanced by the inclusion of antigen. PBMC from volunteer donor D1 were cultured as described in Fig. 1 in the absence or presence of 10 ug/ml flu-matrix peptide, IL-2 and IL-12 (10 ng/ml). On day 12, cells were stained and analyzed by flow cytometry, staining for CD8 and CD4 as described in Fig. 2. The open bar reflects cultures stimulated with 10 U/ml IL-2 alone; hatched bar, cultures stimulated

with 10 U/ml IL-2 and 10 ng/ml IL-12; gray bar, cultures stimulated with 10 U/ml IL-2 and 10 μg/ml flu-matrix peptide; and black bar, cultures stimulated with 10 U/ml IL-2, 10 ng/ml IL-12, and 10 μg/ml flu-matrix peptide. Similar findings were observed using PBMC from normal donors D2 and D3.

Figure 4: The addition of IL-12 to IL-2 increased flu-matrix peptide-specific IFN-γ secreting precursors from PBMC after IVS on flu matrix peptide. PBMC from normal donors D1, D2, and D3 were cultured with 10 μg/ml flu-matrix peptide as described in Fig. 1 in the presence of 10 U/ml IL-2 alone or 10 U/ml IL-2 and IL-12 (1 or 5 ng/ml). Peptide-specific precursors were measured by IFN-γ ELIspot analysis following restimulation of the cultured cells with either control media, flu-matrix peptide (panel A), or an EBV HLA-A2 binding 9-mer peptide (panel B). Statistical analysis was performed using the Student's t test; ns=not significantly different, nd=not done. Evaluation of each donor was done independently at different times.

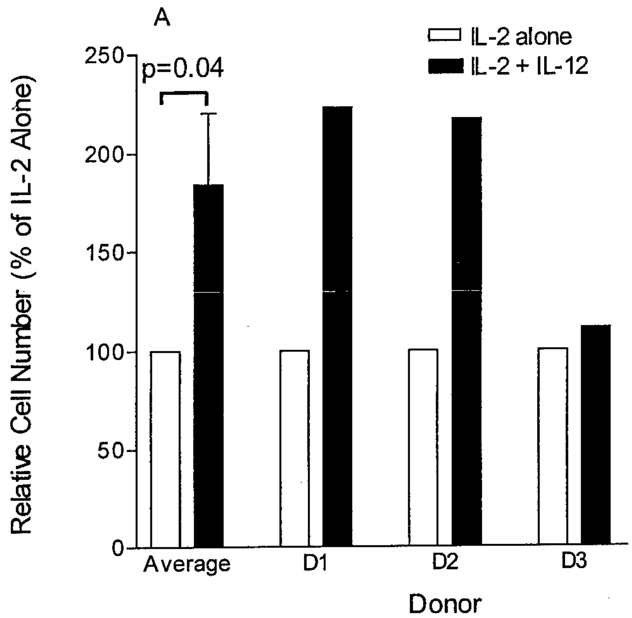
Figure 5. Generation of immunity to HER-2/neu helper peptide, p776-790, in patients with HER-2/neu-overexpressing cancers. Patients were vaccinated with HER-2/neu peptides once a month for 6 months. Antigen-specific immunity was assessed on Days 0 and 150. T cell proliferation was assessed by ^{3}H-thymidine incorporation assays and stimulation index was calculated from 24-well replicates. p776-790 (immunizing peptide) response is depicted by black bars and p42-56 (irrelevant, non-immunizing, HER-2/neu peptide) response is depicted by the open bars. Statistical analysis was performed using the Student's t test.

Figure 6: The addition of IL-12 to IL-2 increases total cell yield after IVS with HER-2/neu helper peptide using PBMC derived from cancer patients. Data shown is the average (± SEM) and individual increase in cell numbers for all 3 patient donors, 1276, 6622, and 0982 from cultures stimulated with 10 μg/ml HER-2neu peptide p776-790 with 10 U/ml IL-2 alone (open bars) or with 10 ng/ml IL-12 and 10 U/ml IL-2 (filled bars) as described in Materials and Methods.

Figure 7: The addition of IL-12 to IL-2 had no effect on CD8/CD4 ratios after IVS with HER-2/neu helper peptide using PBMC derived from cancer patients. PBMC from patient donors (1276, 6622, 0982) were cultured in 10 μg/ml p776-790 and either 10 U/ml IL-2 alone or 10 U/ml IL-2 and 10 ng/ml IL-12 and analyzed for phenotype by flow cytometry. Data shown are obtained from a minimum of 5000 gated events. Shown are the CD8/CD4 ratios of cultures stimulated with antigen and IL-2 alone (open bars) and IL-2 + IL-12 (black bars).

Figure 8: IL-12 in combination with IL-2 increases peptide-specific TNF-α release from cultures after IVS with HER-2/neu helper peptide using PBMC derived from cancer patients. PBMC from patient donors (1276, 6622, and 0982) were cultured with HER-2/neu helper 15-mer peptide, p776-790 and either 10 U/ml IL-2 alone or 10 U/ml IL-2 and 10 ng/ml IL-12. The cells were subsequently tested for TNF-α release following restimulation with no antigen, 10 μg/ml p776-790 or 10 μg/ml p42-56. Each determination is the average (± SEM) for

triplicate determinations. The absence of a bar indicates cytokine levels below the limits of detection of the assay.


A
IL-2 alone
IL-2 + IL-12
p=0.04
Relative Cell Number (% of IL-2 Alone)
250
200
150
100
50
0
Average
D1
D2
D3
Donor

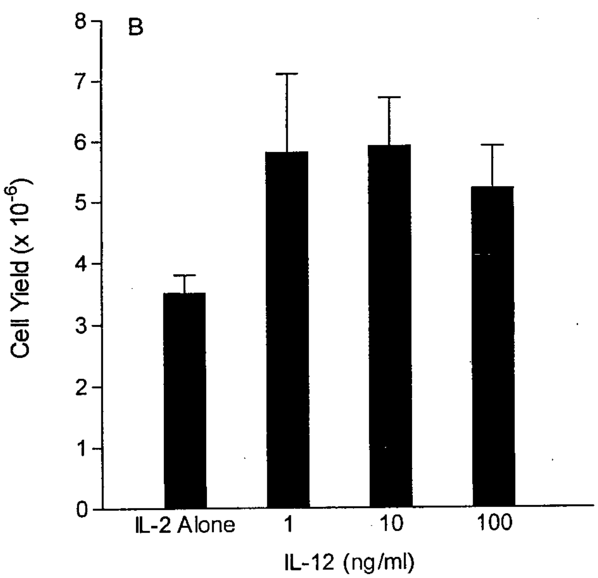

B
Cell Yield (x 10-6)
8
7
6
5
4
3
2
1
0
IL-2 Alone
1
10
100
IL-12 (ng/ml)

Figure 1

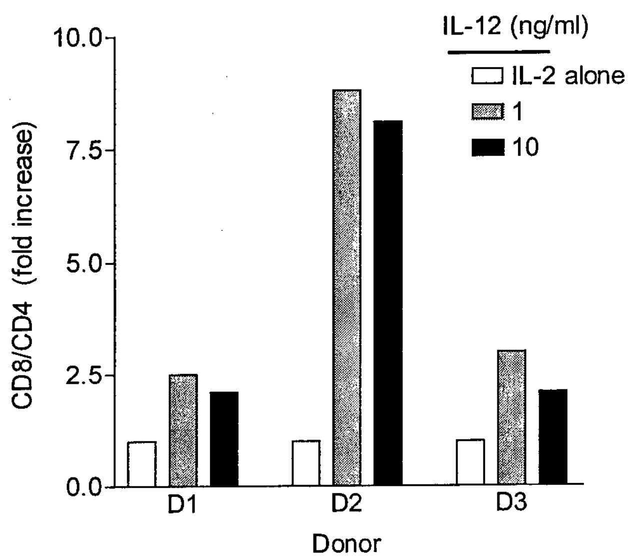

IL-12 (ng/ml)
IL-2 alone
1
10
10.0
7.5
5.0
2.5
0.0
CD8/CD4 (fold increase)
D1
D2
D3
Donor

Figure 2

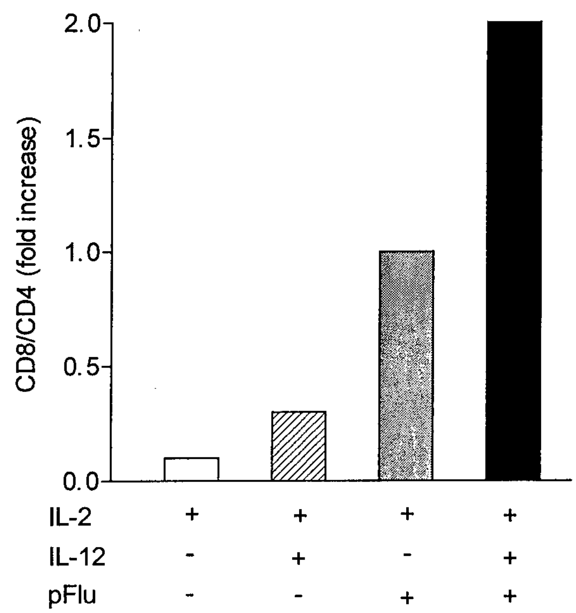


Figure 3

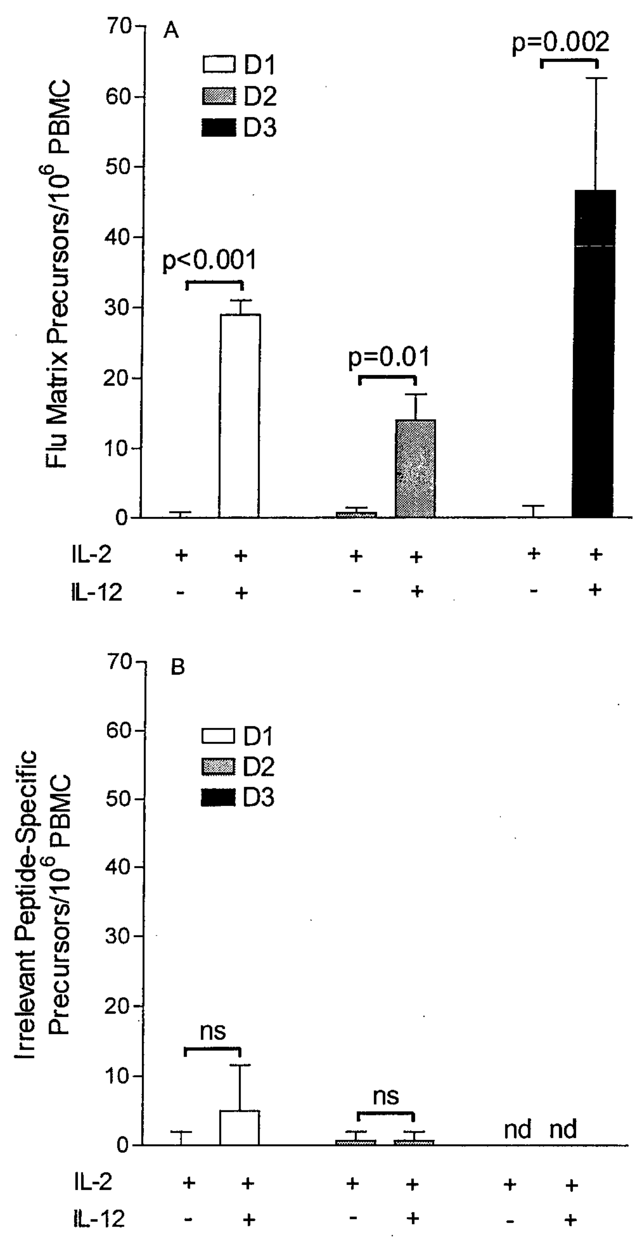
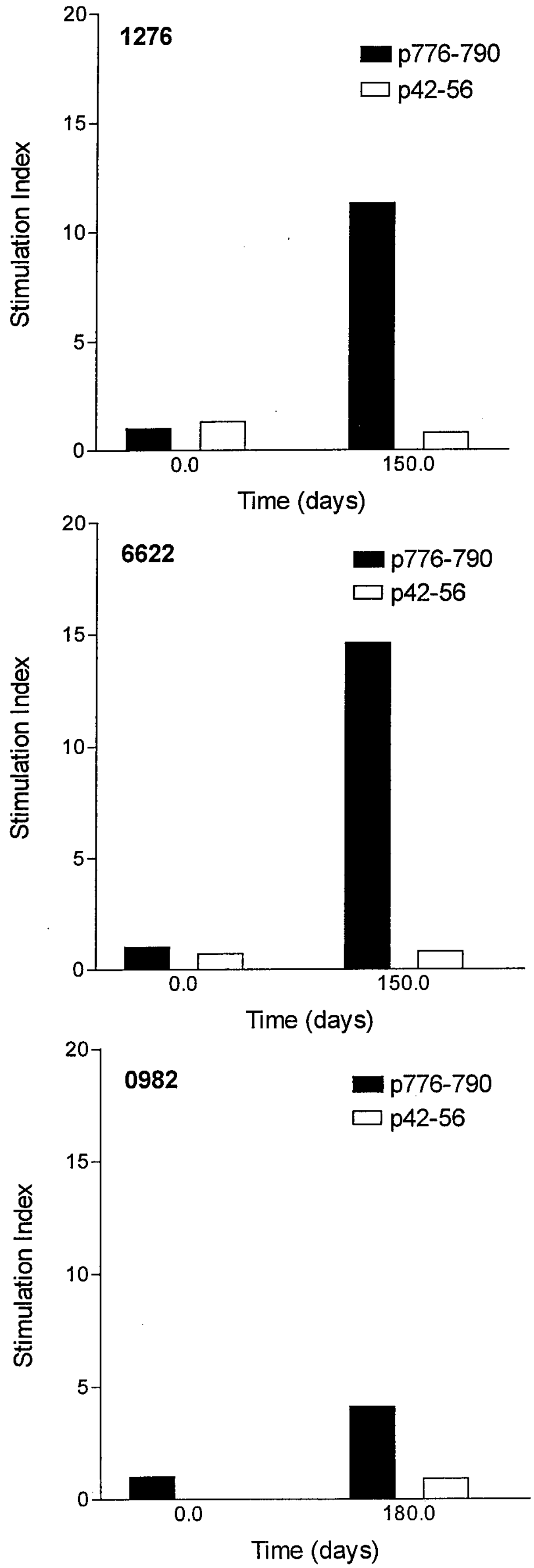
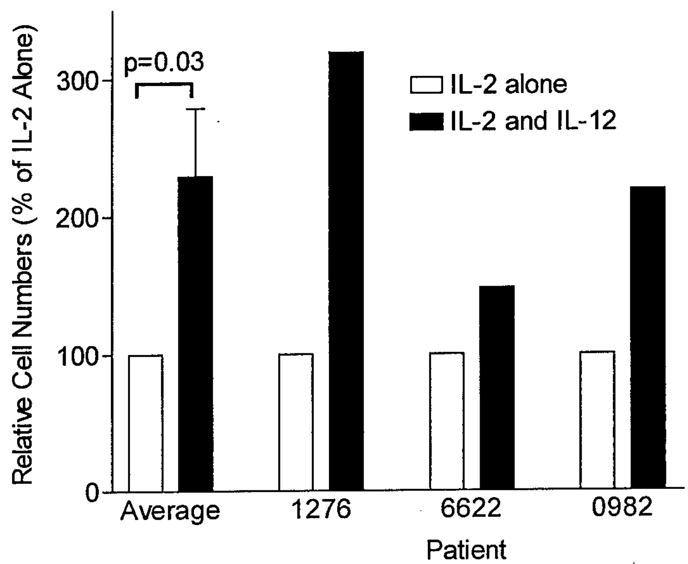

A
70
60
50
40
30
20
10
0
Flu Matrix Precursors/10⁶ PBMC
D1
D2
D3
p<0.001
p=0.01
p=0.002
IL-2 + + + + + +
IL-12 - + - + - +
B
70
60
50
40
30
20
10
0
Irrelevant Peptide-Specific Precursors/10⁶ PBMC
D1
D2
D3
ns
ns
nd nd
IL-2 + + + + + +
IL-12 - + - + - +

Figure 4



1276
p776-790
p42-56
Stimulation Index
20
15
10
5
0
0.0
150.0
Time (days)
6622
p776-790
p42-56
Stimulation Index
20
15
10
5
0
0.0
150.0
Time (days)
0982
p776-790
p42-56
Stimulation Index
20
15
10
5
0
0.0
180.0
Time (days)

Figure 5



Relative Cell Numbers (% of IL-2 Alone)
300
200
100
0
p=0.03
IL-2 alone
IL-2 and IL-12
Average
1276
6622
0982
Patient

Figure 6

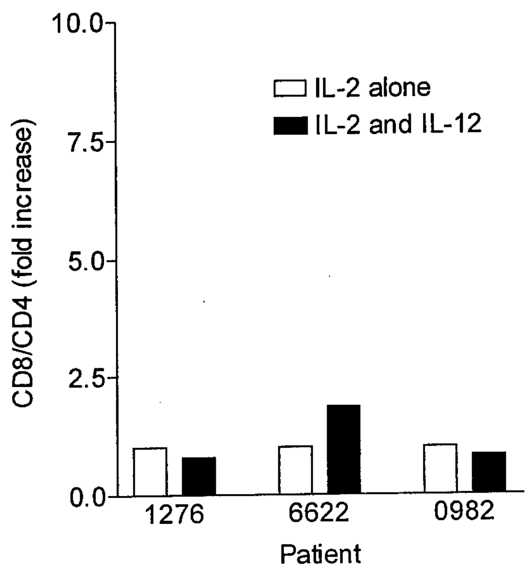
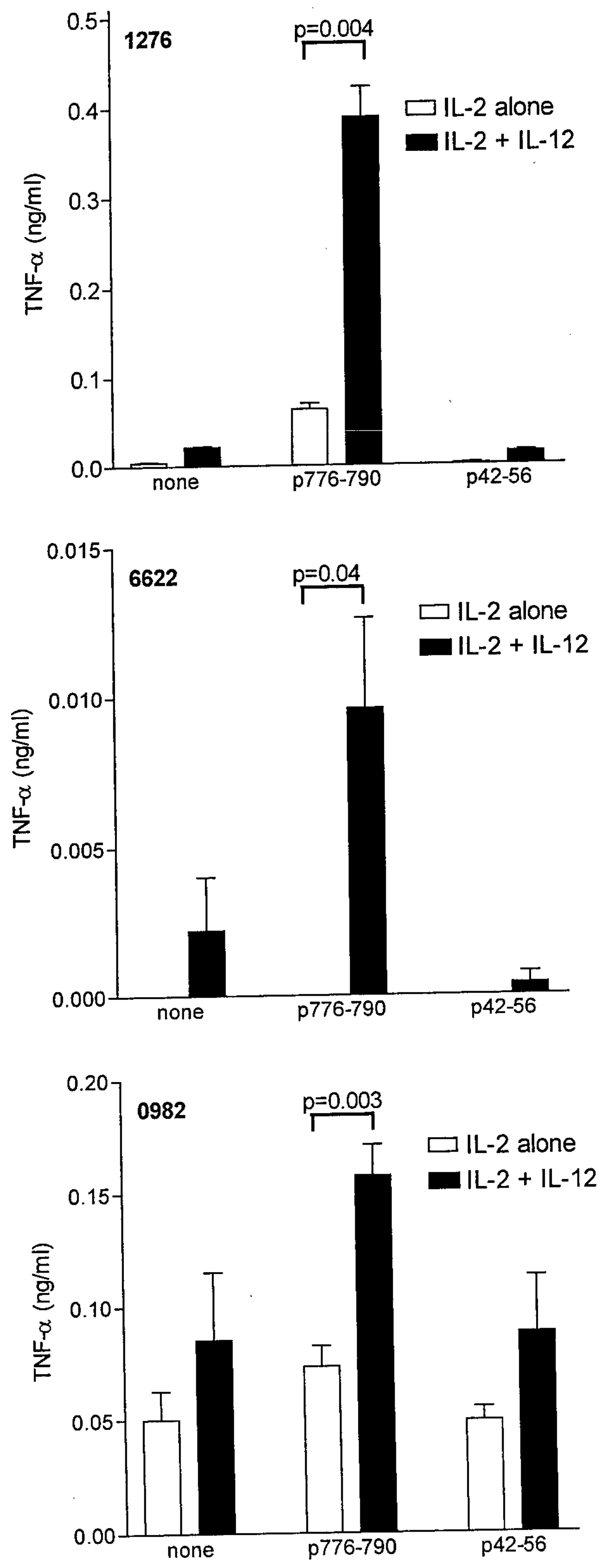

10.0
7.5
5.0
2.5
0.0
CD8/CD4 (fold increase)
IL-2 alone
IL-2 and IL-12
1276
6622
0982
Patient

Figure 7



1276
p=0.004
IL-2 alone
IL-2 + IL-12
TNF-α (ng/ml)
0.5
0.4
0.3
0.2
0.1
0.0
none
p776-790
p42-56
6622
p=0.04
IL-2 alone
IL-2 + IL-12
TNF-α (ng/ml)
0.015
0.010
0.005
0.000
none
p776-790
p42-56
0982
p=0.003
IL-2 alone
IL-2 + IL-12
TNF-α (ng/ml)
0.20
0.15
0.10
0.05
0.00
none
p776-790
p42-56

Figure 8

Diversity of the T cell repertoire responding to a dominant HLA-A2 epitope of HER-2/neu[1]

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Short Title: Diversity of the T cell Repertoire

Keywords: peptide-based vaccines, T cell repertoire, cytolytic T cells, T cell clone

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Abbreviations: BLCL, B lymphoblastic cell line; TCR, T cell receptor; CTL, cytolytic T lymphocyte

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Abstract

T cell clones specific for HER2/neu HLA-A2 peptide p369-377 were isolated from an ovarian cancer patient who had been vaccinated with HER2/neu helper epitopes that contain HLA-A2-binding CTL epitopes within their sequences. Throughout the course of immunization, PBMC from this patient showed strong proliferative responses to the HER2 helper epitope p369-384. Following vaccination, T cell clones specific for p369-377 were isolated by limiting dilution and then characterized. The responding T cell repertoire generated was both phenotypically and functionally diverse. A total of 21 p369-377 clones were generated from this patient. With the exception of two clones, all clones were $CD3^+$. Sixteen of the clones were $CD8^+/CD4^-$. Five of the clones were $CD4^+/CD8^-$, despite being generated with an HLA-A2-binding peptide. Nineteen of 21 of clones expressed the αβ–TCR. The remaining 2 clones expressed the γδ TCR. Selected αβ–TCR clones could lyse HLA-A2-transfected HER2-overexpressing tumor cells and p369-377-loaded BLCL. In addition to their lytic capabilities these clones could be induced to produce interferon-γ (IFN-γ) specifically in response to p369-377 peptide stimulation. The 2 γδ-TCR clones expressed CD8 and lysed HLA-$A2^+$ HER-2/neu^+ tumor cells, but not HLA-$A2^-$ HER-2/neu^+ tumor cells. One of γδ-TCR clones also released peptide directly in response to p369-377 stimulation. These results suggest that a tumor antigen T cell response, directed against a specific epitope, can be markedly polyclonal at multiple levels including CD4/CD8 and TCR.

Introduction

Understanding the immune response to tumor antigens is critical for the design and implementation of immunotherapeutic techniques such as cancer vaccination and adoptive immunotherapy. It is currently thought that the cell-mediated immune response is a critical immune effector arm involved in tumor eradication. In particular, the $CD3^{+}/CD8^{+}$ cytolytic T lymphocyte response has been studied extensively. Many tumor antigens have been discovered over the last decade from which HLA-class I epitopes have been identified [1]. To activate and expand anti-tumor cytolytic T cells, these HLA-class I epitopes have been used directly in native form for vaccination. However, this vaccination strategy often fails to elicit an effective immune response. In addition, the peptide-specific T cells elicited may fail to recognize tumor. For example, the HER-2/neu HLA-A2 peptide, p369-377, initially described by Fisk and colleagues as an immunodominant epitope of HER-2/neu was used as a vaccine in a phase I clinical trial when it was observed that patients readily elicited responses following immunization. However, in all patients the CTL elicited were unable to recognize $HLA-A2^{+}$ $HER-2/neu^{+}$ tumor cells [2]. More recently, we have reported that when p369-377 is naturally processed and presented during vaccination, peptide-specific T cells can recognize and lyse HLA-A2-matched HER-2/neu-overexpressing tumor cells [3]. These contrasting findings may suggest that different populations of effector T cells can be elicited to the same peptide and that the resulting T cell repertoire may depend on how the antigen is processed by the immune system. Recently, interest in developing strategies to mimic natural processing of antigen has increased.

In this study, we examined the functional phenotypic diversity of the T cell repertoire to HER-2/neu peptide p369-377 in a HLA-A2 patient with a HER-2/neu-overexpressing ovarian cancer. The patient had been previously immunized with a HER-2/neu helper peptide vaccine, which contained the helper peptide p369-384 [3]. This helper peptide encompassed the HLA-A2 peptide, p369-377. Therefore, the resulting p369-377-specific T cell repertoire produced *in vivo* was a function of natural processing of antigen.

Results

Immunity to HER-2/neu peptide p369-384 is generated as a result of vaccination with helper peptides in an HLA-A2$^+$ ovarian cancer patient. T cell proliferative responses were measured against 15-aa HER-2/neu peptides prior to, during and following the vaccination series. As shown in Figure 1A, prior to immunization proliferative responses were not detected to any of the HER-2/neu peptides contained within the vaccine formulation. The stimulation index to p369-384 was 1.0, to p688-p703 was 0.9, and to p971-984 was 0.8. The patient did demonstrate a proliferative response to tetanus toxoid (S.I.=7.5). Following vaccination, a T cell proliferative response was measured against 15-aa HER-2/neu peptide, p369-384 (Figure 1B). The stimulation index to p369-384 was 4.2, to p688-p703 was 1.4, and to p971-984 was 1.6. The tetanus toxoid response remained stable during the course of immunization and after the last vaccine was 4.9. The peptide, p369-384, encompassed within its sequence a defined HLA-A2 binding epitope, p369-377 [3,4]. Twenty-one T cell clones were isolated using this HLA-A2 peptide, representing a cloning efficiency of approximately 5%.

A polyclonal T cell response could be elicited to p369-377, an HLA-A2 HER-2/neu epitope encompassed with the sequence of the helper epitope, after active immunization. The majority (19/21) of the clones were greater than 90% CD3$^+$. Nineteen of 21 of the clones expressed the αβ-TCR, and 2 expressed γδ-TCR (clones IDI and 3F7). Representative histograms of TCR staining for 2 αβ-TCR (example clones 2F10 and 2G2) and the 2 γδ-TCR T cell clones are shown in Figure 2. Although the majority (16/21) of the monoclonal populations expressed CD8, some clones expressed (5/21) CD4 despite being cloned using the HLA-A2 peptide, p369-377. Representative flow cytometry dot plots from 4 αβ-TCR clones, 2F10 (CD8$^+$), 2G2 (CD8$^+$), 2A2 (CD4$^+$), and 3G9 (CD4$^+$) stained with anti-CD8 and anti-CD4, are shown in Figure 3. Representative clones from each phenotypic group were chosen for evaluation in a more detailed fashion.

HER-2/neu peptide-specific αβ-TCR clones secrete IFN-γ in response to antigen. The αβ-TCR clones were examined for peptide-specific release of IFN-γ in an ELIspot assay (Figure 4). Representative αβ-TCR, CD8+ T cells were tested against p369-377, an irrelevant HER-2/neu 9 amino acid peptide, or no antigen. The number of spots detected in wells containing p369-377 was 137 ± 17 (mean ± s.e.m.), 177 ± 22, and 102 ± 17, for the clones, 3H8, 2G2, and 2F6 respectively. These means were greater than the mean number of spots detected in wells containing either no antigen (3H8, 45 ± 4, p=0.003; 2G2, 63 ± 10, p=0.002; 2F6, 54 ± 15, p=0.02) or irrelevant peptide (3H8, 50 ± 7, p=0.03; 2G2, 59 ± 19, p=0.03; 2F6, 51 ± 15, p=0.05). These clones also all released IFN-γ into the supernatant as assessed by ELISA (data not shown).

HER-2/neu peptide-specific αβ-TCR clones lyse HLA-A2$^+$, HER-2/neu$^+$ tumor cells. The clones were examined for lysis of peptide-loaded autologous BLCL or HER-2/neu-expressing tumor cell lines. As an example, 2 CD3$^+$CD8$^+$CD4$^-$ clones (2G2 and 2G7) and 1 CD3$^+$CD8$^-$CD4$^+$ (3G9) are shown in Figure 5. The clones examined all demonstrated detectable lysis of p369-377-loaded BLCL and HLA-A2$^+$ HER-2/neu-expressing tumor cells. At an E:T ratio of 40:1, the % specific lysis was 9%, 12% and 13% for clones 2G2 (Panel A), 2G7 (Panel B), and 3G9 (Panel C), respectively against p369-377-loaded autologous BLCL. Responses against non peptide-loaded BLCL were <4% for all clones tested. Against the HLA-A2+, HER-2/neu-expressing SKOV3 tumor cell line, the % specific lysis at 40:1 E:T was 7% , 7%, and 6% for 2G2 (Panel A) , 2G7 (Panel B), and 3G9 (Panel C), respectively. Lysis of parental, HER-2-expressing SKOV3 tumor cells was not detectable for any of the tested clones.

γδ-TCR clones, IDI and 3F7, lyse HLA-A2^{+}, HER-2/neu-expressing tumor cells. The γδ-TCR clones were predominantly CD8 (Figure 6) and were examined for peptide-specific release of IFN-γ against p369-377, an irrelevant HLA-A2 HER-2/neu peptide, or no antigen in an ELIspot assay (Figure 7). The mean number of spots detected in wells containing p369-377 was 57 (± 7, s.e.m.) and 6 (± 1, s.e.m.) for 1D1 and 3F7 respectively. The mean number of spots, in the presence of p369-377, for 1D1 was significantly higher than the mean number of spots detected in wells containing either no antigen (19 ± 4, s.e.m., p=0.015) or irrelevant peptide (26 ± 8, s.e.m., p=0.03). Clone 3F7 did not demonstrate significantly elevated IFN-γ release in response to peptide.

The γδ-TCR clones were also examined for lysis of HER-2/neu-expressing cells lines (Figure 8). Both clones displayed lysis of the HLA-A2^{+}, HER-2/neu-expressing tumor cell line SKOV3-A2. At an E:T ratio of 40:1, the % specific lysis was 20% and 25% for 1D1 (Panel A) and 3F7, respectively (Panel B). Lysis of parental HER-2-expressing SKOV3 tumor cells was less than 1% for both clones at all E:T ratios examined.

Conclusions

A common method to vaccinate patients to generate cytotoxic T cells specific for tumor antigen is to use peptides formulated to bind specifically to HLA class I molecules. Some clinical studies using this strategy have shown that peptide-specific precursor frequencies can become elevated but that these peptide-specific T cells may not recognize antigen naturally processed *in vivo*. The lack of recognition of antigen presented in the MHC may be due to the fact that the peptides, usually constructed from motif-based algorithms, were not naturally processed. Alternatively, the high pharmacological levels of peptides usually administered in a vaccine could have elicited T cell clonal populations that cannot respond to the low levels of peptides presented naturally. Many strategies have been developed to deliver peptides to the inside of the cell where direct binding of the peptide to the newly formed HLA molecules can be achieved; mini-gene epitope delivery [5] or fusing the epitopes with molecules, such as lysosomal membrane associated protein, designed to deliver the peptides to the endosomal pathway [6]. Another strategy is to deliver the HLA class I peptides that are fully contained within longer helper epitopes, which can result in natural presentation of the encompassed class I peptides.

We immunized patients with T helper epitopes derived from HER-2/neu which encompassed known HLA-class I motifs to generate HER-2/neu-specific CTL in cancer patients [3]. Clinical vaccination with longer peptides encompassing HLA-class I binding motifs allows exogenous uptake of helper epitopes within the HLA-class II processing pathway. Theoretically, once internalized and processed, peptides may be available to the HLA-class I processing pathway for presentation [7]. Thus, the generation of HLA-class I-restricted T cells may represent an immune response that required internal processing and presentation of antigen rather than exogenous peptide loading. We cloned T cells from patient successfully immunized with T helper peptides derived from HER-2/neu to evaluate the repertoire of the HLA-class I peptide-specific response that would have been elicited by endogenous processing. The studies described here demonstrate diversity of the repertoire, which shows a polyclonal peptide-specific T cell response consisting not only of $CD4^+$ and $CD8^+$ T cells, but also $\alpha\beta$-TCR and $\gamma\delta$-TCR T cells specific for HER-2/neu peptide antigen.

Isolating several T cell clones specific for the HER-2/neu 9-mer peptide, p369-377, we identified both $CD4^+$ T cell clones as well as $CD8^+$ T cell clones. As expected, the majority of the clones isolated were $CD8^+$. The CD4 molecule, by binding to HLA-class II, is known to play a critical role in stabilizing the interaction of HLA class II:peptide with the TCR. The presence of CD4 may promote the expansion of low-affinity peptide-specific TCRs and ensure a diverse T cell response. Although CD4 is predominantly associated with regulating the immune response to longer peptides in association with HLA class II, at lower frequencies CD4 plays a role in regulating HLA class I-restricted T cells. HLA-class I-restricted CD4+ T cells have been associated with several malignancies such as melanoma, colon, and pancreatic cancer [8,9]. The frequency of CD4 cytolytic T cells responding to a 9-mer peptide epitope is typically low. Our finding that nearly 25% of the T cells cloned using p369-377 were $CD4^+$ suggests that this phenotype may represent a substantial proportion of the *in vivo* repertoire of HER-2/neu-specific T cells. Alternatively, the *in vitro* culture conditions may have favored expansion of $CD4^+$ T cells rather then $CD8^+$ T cells. Although MHC class I-restricted CD4 cells have been associated with many different tumor types, their role in the anti-tumor immune response is not well understood. A study by Yang and Wilson demonstrated that MHC class I-restricted T cell could be therapeutic. In that study, it was observed that MHC class I-restricted adenovirus specific CD4 T cells could clear viral-infected hepatocytes after adoptive transfer [10].

Most investigations of the T cell response to a dominant HLA-class I epitope generated either by vaccination or natural infection have focused on the $\alpha\beta$-TCR$^+$, CD3$^+$, CD8$^+$ T cells. Typically this population of T cells is polyclonal at the level of Vβ [11,12]. While the predominant TCR used in the present study was the $\alpha\beta$-TCR, 2 of the 21 clones (10%) expressed $\gamma\delta$-TCR. $\gamma\delta$-TCR T cells are involved in a wide range of immune responses to infectious and non-infectious diseases, including malaria, mycobacterial infections, cancers, and autoimmune disorders such as multiple sclerosis [13]. Often, $\gamma\delta$-TCR T cells clones are isolated from the tumor-infiltrating lymphocyte population of many tumors including dysgerminoma [14], seminoma [14], renal carcinoma [15], lung [16], colorectal [17], and melanoma [18]. Many tumor-associated $\gamma\delta$-TCR have a CD8+, cytolytic phenotype similar to those described in the present study [16]. The recruitment to and role of these unique cells in mediating antitumor immunity is mostly unknown. In autoimmune diseases, some of the pathological observations have been attributed to infiltrating $\gamma\delta$-TCR T cells. For example, antibody depletion of $\gamma\delta$-TCR reduces demyelination and inflammation in experimental murine multiple sclerosis [19]. It is unknown if autoreactive $\gamma\delta$-TCR T cells respond secondarily to damaged and stressed tissue[20] or if they initiate autoimmunity directly. One hypothesis [21] is that, given the broad range of regulation by multiple mechanisms of antigen presentation and natural localization to epithelial tissue, $\gamma\delta$-TCR T cells are sentinels for the immune system and are capable of alerting the immune system to the presence of danger (e.g. infection, tumors, etc.).

In summary, the results presented here demonstrate that the T cell repertoire to HER-2/neu peptide, p369-377 is polyclonal both at the level of the T cell subset and the level of the

TCR. A broad and diverse T cell response to antigen in our view would likely be the most effective immune response for the prevention and treatment of cancer. This is potentially achieved through natural processing of antigen rather than exogenous binding of HLA motifs. The presence of multiple T cell subsets would likely minimize the potential for the tumor to evade the immune response since a limited T cell response may be more easily overcome by tumor evasion strategies.

Materials and Methods

Patient: The patient was enrolled in a Phase I HER-2/neu peptide-based vaccine trial approved by the University of Washington's Human Subjects Division and the United States Food and Drug Administration and had received definitive conventional therapy for her disease [3]. The patient signed a protocol-specific consent and received monthly vaccinations with three 15 amino acid (15-aa) HER-2/neu-derived peptides, p369-p384, p688-p703 and p971-p984, containing within each the putative HLA-A2 binding motifs p369-p377 [22], p689-p697 [23], and p971-p979 [24]. The vaccine preparation was prepared and delivered as previously described [3]. The patient underwent peripheral blood draws prior to and 30 days following each vaccination for immunologic monitoring. Leukapheresis was obtained 30 days following the final vaccination for the generation of T cell clones.

Materials: The following peptides used in this study, either for immunization or *in vitro* use, were HER-2/neu peptides, p369-384, KIFGSLAFLPESFDGDPA [25], p688-703, RRLLQETELVEPLTPS [25], p971-984, ELVSEFSRMARDPQ [25], p369-377, KIFGSLAFL, p1066-1074, SEEEAPRSP. All peptides used for *in vitro* immunological monitoring were manufactured either by United Biochemical Inc. (Seattle, WA) or Multiple Peptide Systems (San Diego, CA) and all were greater than 95% pure as assessed by HPLC and mass-spectrometric analysis. Ficoll/Hypaque was purchased from Amersham Pharmacia Biotech (Upsalla, Sweden). RPMI-1640, HBSS, and PBS were purchased from Life Technologies (Rockville, MD) and EHAA-120 from Biofluids (Rockville, MD). [^{3}H] thymidine and [^{51}Cr] sodium chromate were purchased from NEN Life Science Products (Boston, MA), human AB+ serum from Valley Biomedical, Incorporated (Winchester, VA), sterile nitrocellulose-backed microfiltration 96-well plates from Millipore Corp (Bedford, MA), and streptavidin-alkaline phosphatase and AP-colorimetric reagents were from BioRad (Hercules, CA). Purified anti-IFN-γ (clone # 1-D1K) and biotin-conjugated anti-IFN-γ (clone # 7-B6-1) were purchased from Mabtech AB (Nacka, Sweden). Anti-CD8-FITC, anti-CD4-PE, anti-$\gamma\delta$-TCR, and anti-$\alpha\beta$-TCR antibodies were purchased from Pharmingen (San Diego, CA). HLA testing was performed by the Puget Sound Blood Bank (Seattle, WA).

Cell Lines: EBV-transformed lymphoblastoid cells (BLCL) were produced from peripheral blood mononuclear cells (PBMC) using culture supernatant from the EBV-producing B95-8 cell line (American Type Culture Collection, Manassas, VA). The HER-2/neu-overexpressing cell lines SKOV3 and SKOV3-A2, and BLCLs were maintained in RPMI-1640 with L-glutamine, penicillin, streptomycin, 2-mercaptoethanol and 10% fetal calf serum. The SKOV3-A2 tumor cells are the same as SKOV3 tumor cells, except that they are stably transfected with a vector encoding HLA-A2 [26].

Preparation of PBMC and cloning of peptide-specific T cells: PBMC were isolated by density gradient centrifugation as previously described [25]. Cells were analyzed immediately or aliquoted and cryopreserved in liquid nitrogen in freezing media (90% fetal bovine serum and 10% dimethylsulfoxide) at a cell density of 25-50 x 10^6 cells/ml until use. Antigen-specific T cell clones were generated by culturing 25 x 10^6 PBMC in T25 tissue culture flasks in 20 mls of T cell medium. The HLA-A2 peptide, p369-377, was added to the flasks to 1 μm. The flasks were incubated at 37^0C and 5% CO_2. On day 3 and every other subsequent day, IL-2 was added to 5 U/ml. On day 10, *in vitro* stimulation was performed with peptide-pulsed, irradiated autologous PBMC. The cultures were further incubated for an additional 10 days with periodic IL-2 administration. The T cell line was cloned after 2 *in vitro* stimulations. For cloning, bulk cultures were diluted to achieve approximately 0.3 viable cells/200 μl and plated onto 4 flat-bottom 96-well plates in complete medium. Peptide-pulsed, irradiated autologous PBMC (2.0 x 10^5) were added to each well in the presence of 50 U/ml IL-2. The wells were then tested for lytic activity in a ^{51}Cr release assay using 50 μl of cells from each well after 14 days. Positive wells were identified as those having specific-activity of 5% or greater. The positive wells were transferred to new 96-well plates and subsequently restimulated with peptide-pulsed, irradiated autologous BLCL. The cultures were eventually expanded and carried using IL-2 and peptide-pulsed, irradiated autologous BLCL.

T cell proliferation assays: HER-2/neu-specific T cell proliferative responses were measured at baseline and at the end of the study. T cell proliferation was assessed using a modified limiting dilution assay designed for detecting low frequency lymphocyte precursors based on Poisson distribution as previously described [25,27]. Data is reported as both counts per minute (CPM) and as a stimulation index (S.I.), which is the mean of 24 experimental wells divided by mean of 24 no antigen wells. An age-matched control population of 30 volunteer blood donors was analyzed similarly (data not shown). No volunteer donor had a response to HER-2/neu peptides. The mean and 3 standard deviations of the volunteer donor responses to all antigens (S.I. of 1.98) established a baseline, therefore an S.I. of > 2 was considered consistent with an immunized response.

Enzyme-linked immunosorbent spot (ELIspot): An ELIspot assay was used to determine precursor frequencies of peptide-specific CD8+ T lymphocytes as previously described with some minor modifications [3]. On day 1, T cell clones (4 replicates per condition) were plated into 96-well, anti-IFN-γ-coated nitrocellulose plates in 100 μl media. The cells were stimulated with 100 μl of media containing 2.0 x 10^5 autologous, irradiated (3000 rads) BLCLs prepulsed (1 hr, RT) with or without antigen (10 μg/ml). The cells were further incubated for 20 hr at 37^0C and detection of bound IFN-γ was performed as previously described [3].

[^{51}Cr]-release assays: Cytolytic activity was measured using standard 4-hour [^{51}Cr]-release assays as previously described [3]. The percent specific activity was calculated using the following equation: % specific lysis=(sample well release-basal release)/(detergent release-basal release).

Flow cytometry: Clones were harvested and washed in FACS staining buffer (PBS containing 20 mM glucose and 0.5% BSA). Cells were stained in 50 μl of FACS staining buffer containing control antibody, anti-CD4, anti-CD8, anti-αβ-TCR, anti-γδ-TCR, or anti-CD3, for 1-2 hr at 4°C. Following 2 washes the cells were fixed in PBS containing 1% paraformaldehyde and analyzed by flow cytometry. Data presentation and statistical analysis was completed using CellQuest flow cytometry software (BD Immunocytometry Systems, San Jose, CA).

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Figure Legends

Figure 1. *Immunity to HER-2/neu peptide, p369-384 is generated as a result of vaccination with helper peptides in an HLA-A2$^+$ ovarian cancer patient.* Shown are the proliferation responses to media alone, the vaccine peptides, p366-384, p689-703, and p971-984. Tetanus toxoid (tt) was added as a positive control. Data from PBMC drawn at the time of the first visit (Panel A) and the last immunization (Panel B). The long horizontal line spanning the width of the graph depicts average CPM of the no antigen wells + 3 S.D. The short horizontal lines within the data symbols represent the mean of the 24 replicates for each condition. The values shown above each data set are the calculated S.I.s.

Figure 2. *Both αβ-TCR and γδ-TCR clones were isolated following HER-2/neu peptide vaccination.* Shown is a representative flow cytometry analysis, staining for either αβ-TCR (top row) or γδ-TCR (bottom row), of select T cell clones. The results are representative of 2 independent experiments yielding similar results.

Figure 3. *Both CD4$^+$ and CD8$^+$ clones were isolated following HER-2/neu peptide vaccination.* Shown is the representative flow cytometry data of p369-377-specific αβ-TCR T cell clones dual-stained for both CD4 and CD8 (top row). Background irrelevant staining for the same clones in shown the bottom row. The results are representative of 2 independent experiments yielding similar results.

Figure 4. *HER-2/neu peptide-specific αβ-TCR clones secrete IFN-γ in response to antigen.* ELIspot data from αβ-TCR clones 3H8, 2G2, and 2F6 are depicted presented as the mean (+/-

s.e.m.; *, $p<0.05$) number of spots calculated from quadruplicate determinations for clones stimulated with p369-377 (black bars), irrelevant peptide (gray bars), or no peptide (white bars).

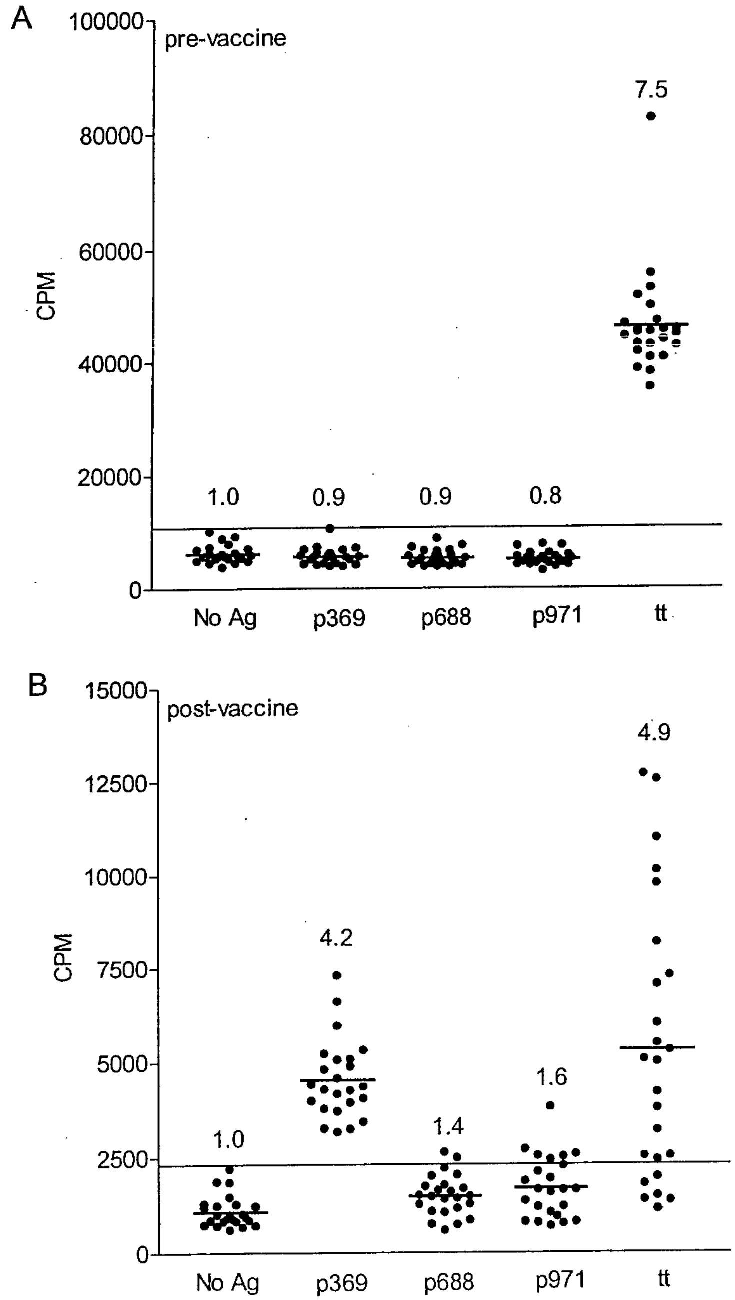
Figure 5. *HER-2/neu peptide-specific αβ-TCR clones lyse HLA-A2$^+$, HER2/neu-expressing tumor cells.* Cytolytic activity data from αβ-TCR clones 2G2 (Panel A), 2G7 (Panel B), and 3G9 (Panel C) are depicted against BLCL-A2 alone (open circles), p369-377-loaded BLCL-A2 (closed circles), or the HER-2/neu-overexpressing tumor cells, SKOV3 (open squares) and SKOV3-A2 (closed squares). The data are presented as the mean (+/- s.e.m.) of triplicate determinations at each of 3 E:T ratios, 40:1, 20:1, and 10:1.

Figure 6. *γδ-TCR T cell clones could be isolated following HER-2/neu vaccination.* Shown is the representative flow cytometry data of p369-377-specific γδ-TCR T cell clones dual-stained for both CD4 and CD8 (top row). Background irrelevant staining for the same clones in shown the bottom row. The results are representative of 2 independent experiments yielding similar results.

Figure 7. *One of the CD8$^+$ γδ-TCR clones, 1D1 secretes IFN-γ in response to antigen.* ELIspot data from γδ-TCR clones 1D1 and 3F7 are depicted and presented as the mean (+/- s.e.m.; *, $p<0.05$) number of spots calculated from quadruplicate determinations for clones stimulated with p369-377 (black bars), irrelevant peptide (gray bars), or no peptide (white bars).

Figure 8. *The CD8$^+$ γδ-TCR clones displayed HLA-A2-restricted, HER2$^+$ tumor cell lysis.* Cytolytic activity data from γδ-TCR clones 1D1 (Panel A) and 3F7 (Panel B) are depicted against the HER-2/neu-overexpressing tumor cells, SKOV3 (open circles) and SKOV3-A2

(closed circles). The data are presented as the mean (+/- s.e.m.) of triplicate determinations at each of 3 E:T ratios, 40:1, 20:1, and 10:1.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p369
p688
p971
tt
B
post-vaccine
CPM
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12500
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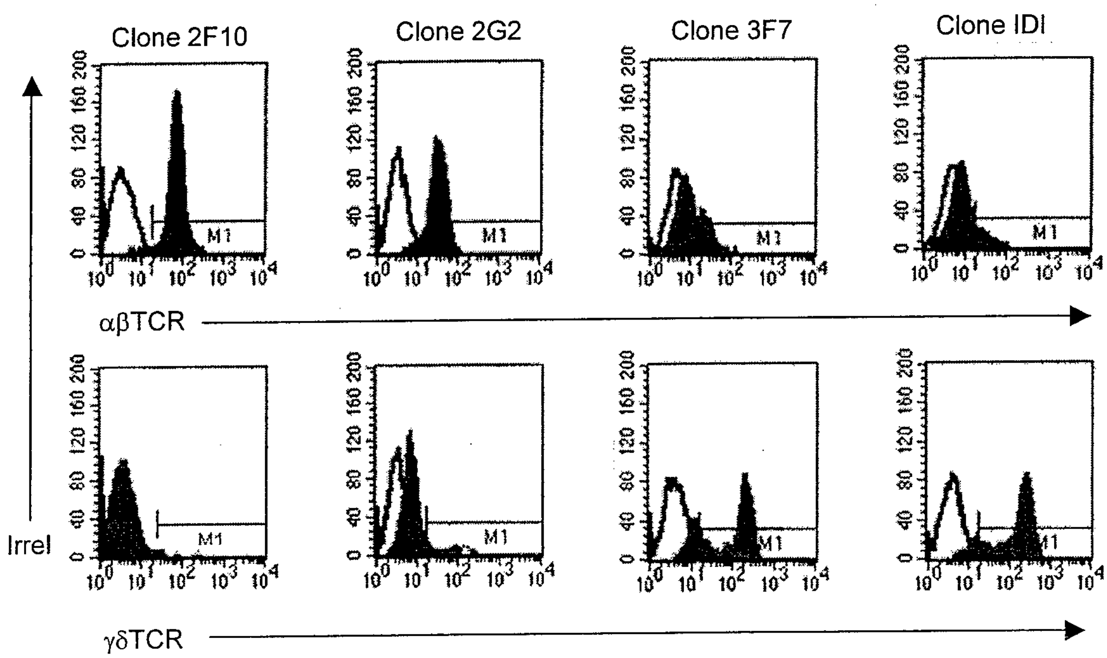
p688
p971
tt

Figure 1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αβTCR
γδTCR
Irrel
M1

Figure 2

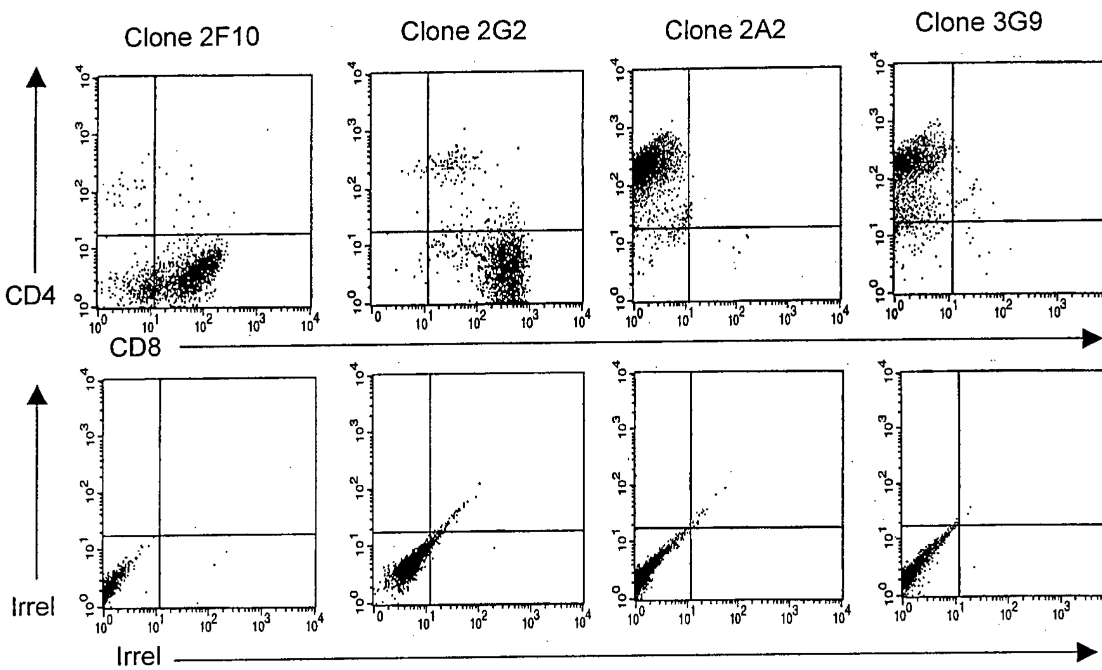


Figure 3

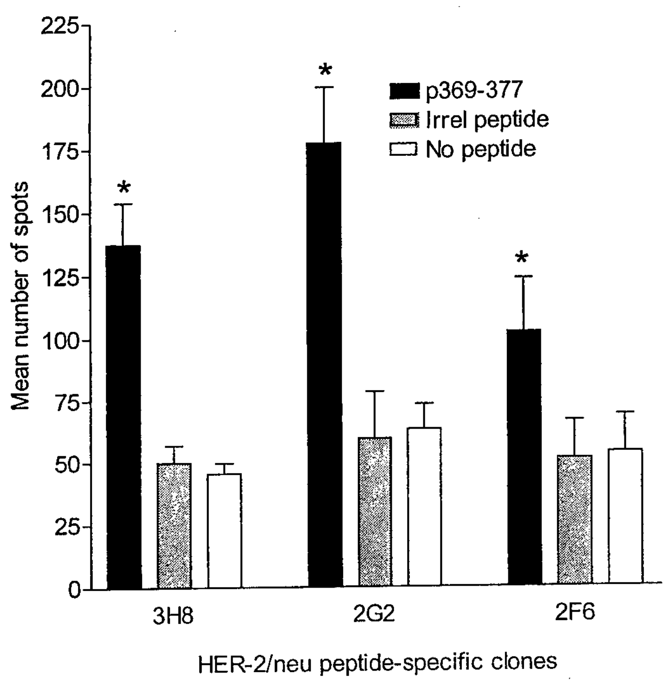
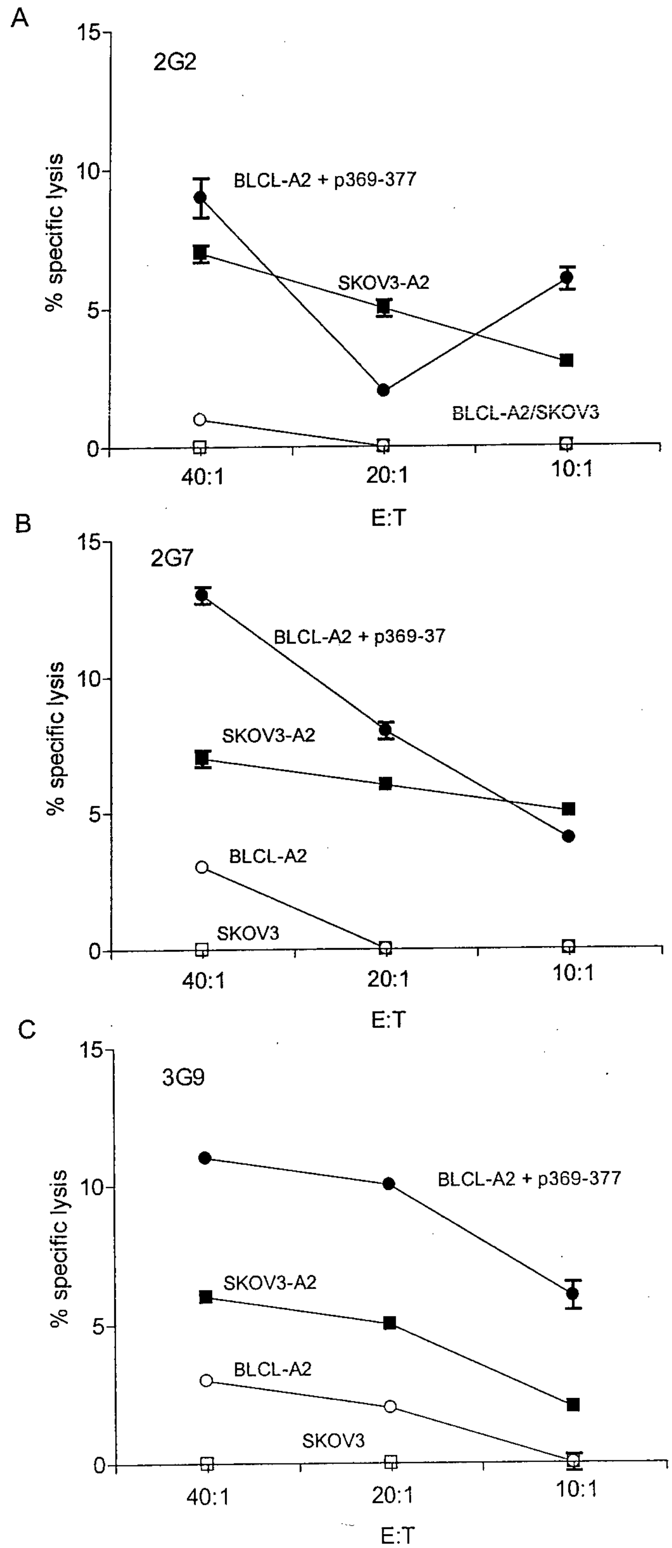

225
200
175
150
125
100
75
50
25
0
Mean number of spots
p369-377
Irrel peptide
No peptide
*
*
*
3H8
2G2
2F6
HER-2/neu peptide-specific clones

Figure 4



A
2G2
BLCL-A2 + p369-377
SKOV3-A2
BLCL-A2/SKOV3
% specific lysis
E:T
B
2G7
BLCL-A2 + p369-37
SKOV3-A2
BLCL-A2
SKOV3
% specific lysis
E:T
C
3G9
BLCL-A2 + p369-377
SKOV3-A2
BLCL-A2
SKOV3
% specific lysis
E:T

Figure 5

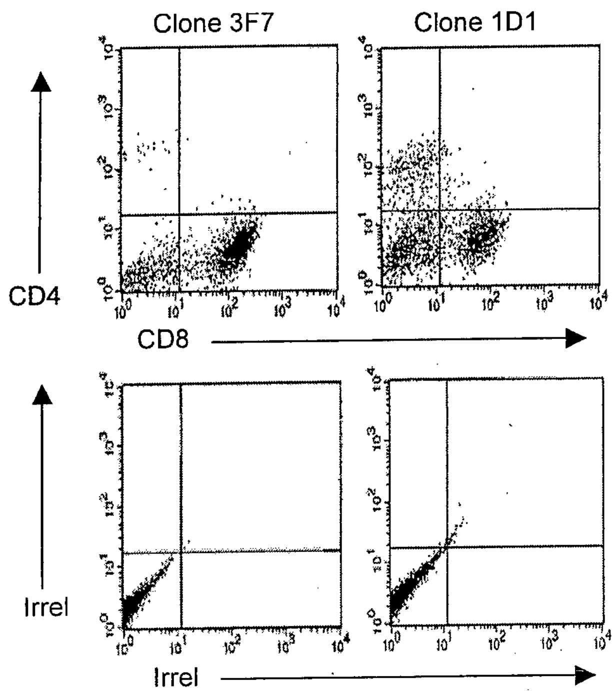
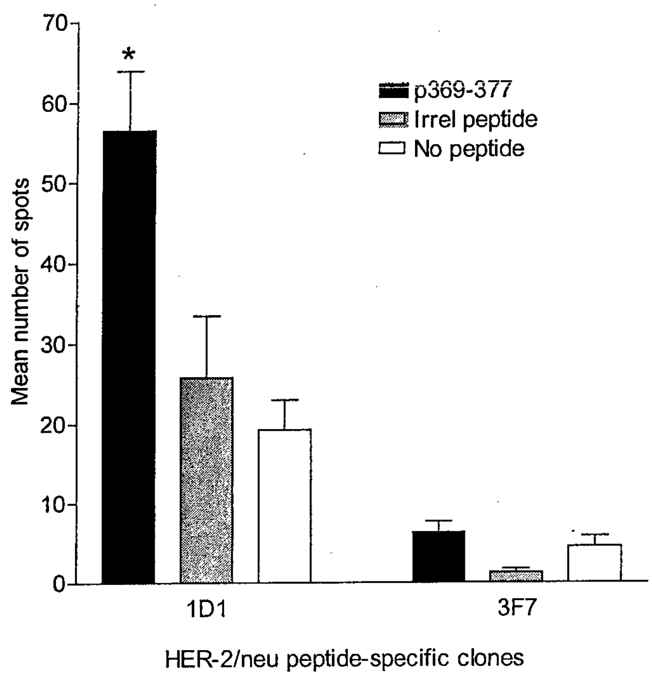


Figure 6



70
60
50
40
30
20
10
0
Mean number of spots
*
p369-377
Irrel peptide
No peptide
1D1
3F7
HER-2/neu peptide-specific clones

Figure 7

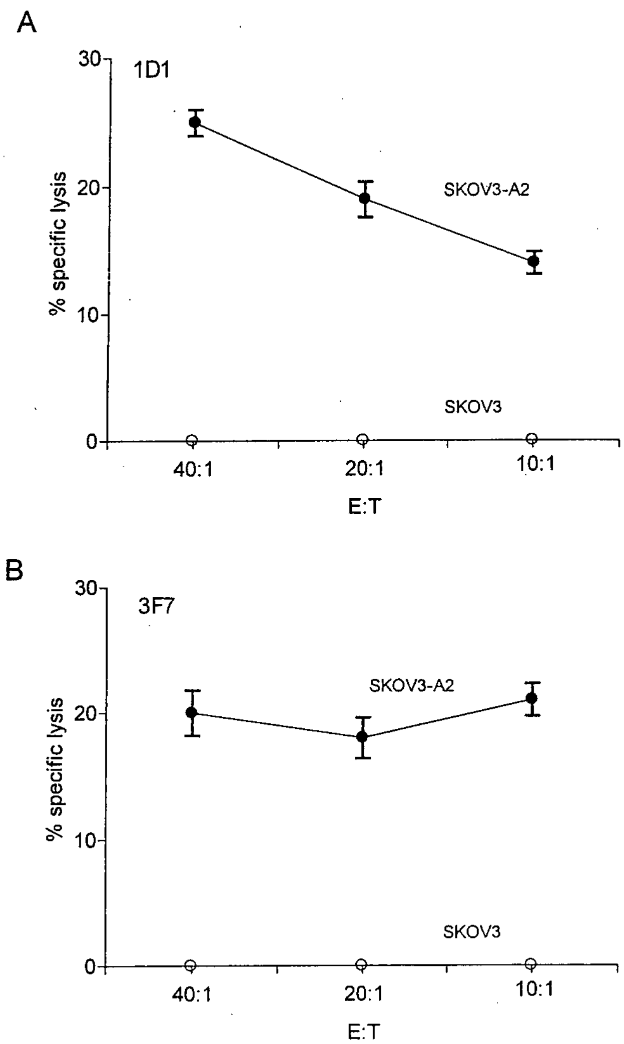

A
30
1D1
20
10
0
% specific lysis
SKOV3-A2
SKOV3
40:1
20:1
10:1
E:T
B
30
3F7
20
10
0
% specific lysis
SKOV3-A2
SKOV3
40:1
20:1
10:1
E:T

Figure 8