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Effector Alloreactive CTL Engineered as Replication-Competent Retrovirus Vector Producer Cells to Deliver Payload to BC Metastatic to the Brain.

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This Idea Proposal was to explore multi-modal immunogene treatment for breast cancer metastatic to brain. The treatments involve using 1) alloreactive cytotoxic T lymphocytes (alloCTL) effector cells that are trained to recognize major histocompatibility antigens (HLA) with 2) replication competent retroviral vectors that code for suicide genes such that infected cells become susceptible to antiviral agents like ganciclovir. We demonstrated that HLA was expressed quite well by several breast cancer cell lines, two of which had a known propensity to metastasize to the brain. We also showed that alloCTL generated by one-way mixed tumor lymphocyte reaction (MLTR) were cytotoxic towards the breast cancer cells and that interferon-gamma is produced upon their contact with relevant target antigens. We know that alloreactivity is involved in this process since antibody to HLA class I inhibits the percentage lysis obtained. We have attempted the knock-down of HLA in the breast cancer cells using siRNA to obtain targets with low, intermediate and high HLA class I expression such that sensitivity to alloCTL can be determined. alloCTL transduced with replication competent retroviral vector (RCR) by spinoculation was most effective and with this technique we achieved up to 35% gene transfer to tumor cells. It was harder to get higher transduction percentages over time as apoptosis was also turned on in the transduced cell population. Finally, we performed a small pilot bioluminescence animal experiment and administered tumor cells with a luciferase marker gene into the brain. After five days, alloCTL was injected right into the center of the tumor or several millimeters away. Histological examination of the brain was performed, and as well, we began working out the technique for detection of CD3+ cells on frozen and fixed brain tissue.
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Introduction
The overall objective of this project is to determine whether alloreactive cytotoxic T lymphocytes (alloCTL), or those transduced with replication competent retrovirus (alloCTL/RCR) can effectively treat breast cancer in the brain. The project was split into two separate categories entailing in vitro work and in vivo work. The specific aims involved an evaluation of 1) the functional characteristics of TCR-transduced human alloCTL in vivo (i.e., cytotoxic activity, proliferation, and production of proinflammatory cytokines upon contact with breast cancer target cells), 2) alloCTL/RCR biodistribution and antitumor effects when infused intracranially into the brains of immunodeficient rodents bearing breast cancer xenografts, and 3) transduction efficiency, replication kinetics, and biodistribution of RCR vectors expressing marker genes after alloCTL-mediated RCR delivery to brains with one or two tumor foci in a breast cancer xenograft model, and 4) overall therapeutic efficacy of alloCTL/RCR treatment to intracranial breast cancer in immunodeficient rodents when the vectors code for suicide genes and prodrug is given.

Statement of Work Performed
Models of Breast Cancer Metastasis to Brain. A very aggressive model for breast cancer brain metastasis, BCM2 BrainG2 had been described and we intended to use this cell line as our primary brain metastasis model. However, since this project was funded we became aware that BCM2 BrainG2 is identical with the MDA-MB-435 cell line and as such likely not breast cancer but melanoma. We decided to move to a different brain-seeking breast cancer model, namely the MDA-MB-231 brain (MDA-MB-231BR) cell line (Yoneda et al, J Bone Miner Res 2001, 16:1486) kindly provided by Dr Toshiyuki Yoneda, UT San Antonio. Cell surface HLA expression of MDA-MB-231BR cells was compared to that of the parental cell line MDA-MB-231 as well as the metastatic subline MDA-MB-231-1833 (obtained from Dr. Joan Massague, Memorial Sloan Kettering). Breast cancer cells were cultured under standard conditions with and without the addition of exogenous interferon-gamma (IFNγ) that is known to increase the expression of HLA class I on the surface of tumor cells. Then adherent breast tumor cells were removed from the flask using 2 mM EDTA containing 1% BSA, washed, stained with FITC-anti-HLA-ABC (W6/32), run on a flow cytometer and analyzed using BD Diva acquisition and analysis software. Data shown in Table I show the percentage of positive cells and the mean fluorescence intensities (MFI) that give the average from two experiments. The data show that the relative antigen densities of HLA class I expressed on the surface of breast cancer cells increases with the addition of rhIFNγ, and that expression is highest in the parental MDA-MB-231 cell line, and slightly reduced in both of the metastatic sublines.

Generation of alloCTL. Based on their high expression of HLA class I, the parental MDA-MB-231 breast cancer cell line was chosen as a stimulator cell for the generation of alloCTL. Assuming that the MDA-MB-231 cell line and sublines share the same HLA type, alloCTL that were generated in a one-way mixed lymphocyte tumor reaction (MLTR) where MDA-MB-231 cells are used as stimulators, should be cytotoxic toward MDA-MB-231 as well as to the brain-seeking sublines. Donors that provide responder PBMC for use in the generation of alloCTL should be HLA-mismatched from the stimulator. To choose a healthy blood donor that would provide PBMC with robust functional cytotoxicity following stimulation with MDA-MB-231, we experimented with three healthy unrelated donors that were serologically HLA typed. The HLA types of the tumor cells relative to the three donors supplying responder PBMC are shown in Table 2.

Table 1. HLA ABC expression on breast cancer cell lines with and without the addition of exogenous IFNγ.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Without IFNg</th>
<th>MFI</th>
<th>% Positive</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>98.0 + 0.05</td>
<td>906 + 1.5</td>
<td>98.5 + 0.38</td>
<td>1402 + 49.7</td>
</tr>
<tr>
<td>MDA-MB-231-1833</td>
<td>95.9 + 0.02</td>
<td>797 + 79.5</td>
<td>96.7 + 0.30</td>
<td>1205 + 91.0</td>
</tr>
<tr>
<td>MDA-MB-231-brain</td>
<td>94.3 + 2.05</td>
<td>717 + 133.0</td>
<td>96.1 + 1.65</td>
<td>1013 + 192.5</td>
</tr>
</tbody>
</table>

PBMC isolated from each of the three donors were then used in a one-way MLTR to generate alloCTL to determine which of the three donors would provide the most robust responder cells following stimulation with irradiated MDA-MB-231 breast cancer cells. In preparation for the one-way MLTR’s, adherent MDA-MB-231 cells were irradiated with 7000 rad by exposure to a Cs 137 source. Stimulator MDA-MB-231 cells were then mixed with the responder PBMC isolated
from the blood of three healthy donors at a 10:1 responder to stimulator (R:S) ratio in AIM-V medium (Invitrogen) containing 60 IU/ml rhIL-2 and 5% responder matched plasma. Lactate production, a measure of cell culture expansion, was measured daily (Figure 1) and lactate concentration was maintained between 500-700 mg/L throughout the culture period by adding fresh medium. Lactate production was similar between the three MLTRs until day 4. However, as the cultures continued, MLTR 3 emerged as the highest producer of lactate indicating that cell growth in this culture was more robust compared to MLTR1 and MLTR2. Twelve days after the start of the MLTRs, alloCTL were counted then restimulated with OKT3. Cells were then cultured until day 14. The numbers of viable cells counted on days 12 and 14 post-MLTRs are shown in Table 2. MLTR 3 had higher viability, and the greatest amount of cell growth over the culture period compared to the other two (MLTR1 and 2).

Key Research Accomplishments

In summary, these data show:

- All tested cells express HLA Class I at a high relative antigen density on the surfaces of the parental breast cancer cell line MDA-MB-231, and sublines MDA-MB-231-1833 and MDA-MB-231-BR
- Surface expression of HLA Class I is slightly higher on MDA-MB-231 than on MDA-MB-231-1833 and MDA-MB-231-BR and the expression (MFIs) can be upregulated by incubation with IFN-γ
- PBMC isolated from donor 3 are the most robust growers following stimulation in a one-way MLTR with irradiated MDA-MB-231 to generate alloCTL.

Based on these findings, PBMC isolated from Donor 3 were used as responder cells to generate alloCTL in one-way MLTR with stimulator MDA-MB-231 breast cancer cells in the following experiments.

Evaluation of the functional characteristics of alloCTL toward breast cancer cells. AlloCTL were generated as described above with PBMC from Donor 3 mixed at a 10:1 R:S ratio with irradiated stimulator MDA-MB-231 cells. On day 15 after one-way MLTR, when alloCTL were evaluated for their cytotoxic ability toward relevant target cells in a 4 hr Cr-51 release assay. Briefly, adherent breast cancer tumor target cells were removed from the flask, loaded with Cr-51 and plated with alloCTL at multiple effector to target (E:T) ratios. After a 4 hour incubation cell death was determined by release of Cr-51 into the supernatant. The data indicate that alloCTL against MDA-MB-231 are cytotoxic toward that cell line, as well as to MDA-MB-231-1833 and –BR (Figure 2a). The ability of alloCTL to kill each of the three target cell lines was similar; there were no significant differences in the percentages of lysis obtained at each E:T. Furthermore, the cytotoxic ability of alloCTL was blocked with an anti-HLA antibody (W6/32, Figure 2b) at the 1:5:1 E:T ratio. Blocking HLA expression resulted in an average of 18.8% ± 1.2% (SEM) reduction in cytotoxicity for each of the three breast cancer target cells. The percentage lysis of MDA-MB-231 and -1833 was significantly reduced (p<0.005) when target cells were incubated with anti-HLA. These data indicate that the cytolytic function of alloCTL is in part HLA-restricted. It is possible that non-HLA restricted killing by T cells or NK cells contributed to the cytotoxicity measured, or CTL directed toward tumor associated antigens, but the degrees would need to be confirmed by inhibition experiments.
The ability of subsets of CD4+ and CD8+ T cells to proliferate and produce IFN-γ following re-stimulation with MDA-MB-231, -1833, or –BR was evaluated. AlloCTL were re-stimulated on day 12 post MLTR at a 10:1 R:S ratio with either of the three cell lines, or left unstimulated and then cultured for 72 hrs. Cells were then stained for CD4 and CD8, and fixed, permeabilized and stained for BrdU and IFN-γ according to protocol (BD BrdU Flow Kit). Cells were phenotyped on a LSR II flow cytometer (BD Biosciences), and analyzed using the program FlowJo. The total percentage of CD8+ T cells was significantly higher for alloCTL that were restimulated with MDA-MB-231, -1833, and –BR compared to cells that were unstimulated (54.8, 56.0, 51.8 vs 30.9, respectively, Figure 3a). Furthermore, the restimulation occurred equally well with parental or brain-seeking sublines. The percentages of CD8+ T cells that incorporated BrdU were significantly higher in CD8+ T cells that were restimulated compared to those that were unstimulated (Figure 3c). In contrast, the total percentages of CD4+ T cells were significantly reduced (p<0.0004) when alloCTL were restimulated with MDA-MB-231, -1833, and –BR compared to those that were not restimulated (14.9, 12.1, 11.8 vs 22.2, respectively; Figure 3b). CD4+ T cells did not proliferate in response to restimulation with any of the breast cancer cell lines, as there were no significant differences in BrdU incorporation between CD4+ T cells that were restimulated and those that were unstimulated (Figure 3c). The data show that restimulation of alloCTL with MDA-MB-231, -1833, and –BR breast cancer cell lines results in a significant shift in T cell subsets that make up the alloCTL culture.

The expression of IFN-γ following 72 hour restimulation with MDA-MB-231, -1833, and -BR compared to cells that were unstimulated was also evaluated. Protein transport was blocked during the last 5 hours of culture to allow the IFN-γ to accumulate intracellularly. Cells were then washed and stained as described above. Expression of IFN-γ was significantly higher (p≤0.01) in CD8+ T cells that were restimulated with MDA-MB-231, -1833, and -BR compared to cells that were unstimulated (64.6, 45.1, 43.8 vs 27.5, respectively, Figure 4). Here the stimulation was more robust with the parental cells versus the sublines, however, indicating the MFIs of class I on the surface may have some influence over the response. In contrast, the production of IFN-γ in CD4+ T cells was not affected following restimulation with any of the cell lines (data not shown). To determine if CD4+ T cells were undergoing apoptosis in response to restimulation, CD4+ T cells were stained for intracellular expression of activated caspase 3. The expression of activated caspase 3 increased 1.8 to 2.3 fold in CD4+ T cells that were restimulated with the breast cancer cell lines compared to CD4+ T cells that were unstimulated (Figure 5).
Figure 3. Dot blots indicating the percentages of CD8+ T cells (a) or CD4+ T cells (b) 72 hours after re-stimulation with breast cancer cell lines. c. Percentages of CD8 and CD4 T cells that are also positive for BrdU. * p < 0.005 by Students T test. Data are representative of 2 separate experiments.

Figure 4. Dot blots showing the percent of CD8+IFNγ+ T cells present 72 hours after alloCTL are re-stimulated with breast cancer cell lines. Numbers in bold represent the percent of CD8+ T cells that are producing IFNγ. Data is representative of 2 separate experiments.

Figure 5. Dot blots showing the percent of CD4+T cells that are positive for activated Caspase 3 72 hours after re-stimulation with breast cancer cell lines.
**Key Research Accomplishments** In conclusion, our in vitro data show that:

- alloCTL generated following one-way ML TR using MD A-MB-231 as stimulator cells are cytotoxic toward MDA-MB-231, -1833, and -BR.
- Cytotoxicity is partially blocked with anti-HLA, indicating alloreactive cells are contained within the alloCTL preparations.
- CD8+ T cells present in alloCTL cultures proliferate and produce IFN-γ in response to restimulation with any of the three breast cancer cell lines, whereas CD4+ T cells do not.
- A larger percentage of CD4+ T cells present in alloCTL cultures are positive for activated Caspase 3 following re-stimulation with breast cancer cell lines indicating that restimulation of these cells results in a higher than normal basal level of CD4+ cell apoptosis.

**Breast cancer cells transduced with RCR vectors.** In theory we first needed to demonstrate that breast cancer cells transduced with RCR coding for suicide genes, such as Herpes simplex virus-thymidine kinase (HSV-TK) were susceptible to an antiviral agent such as ganciclovir. We performed multiple toxicity experiments using breast cancer cells, MDA-MB-231 1833 that were transduced with the ACE-TK RCR vector, and the antiviral agent ganciclovir (GCV) at various concentrations. Representative data are shown in **Figure 6**. Nearly all cells at 0.4 µM or higher concentration were nonviable demonstrating the effectiveness of the antiviral agent against the transduced cells. Furthermore, the tremendous bystander effect from the gene therapy approach was demonstrated using various mixtures of transduced to nontransduced tumor cells (**Figure 7**).

**HLA knock-down with siRNA vector.** Here the idea was to have the ability to obtain populations of breast cancer cells with HLA class I expressions at relative antigen densities of high intermediate and low, such that assessment of the suitability of alloCTL or alloCTL/RCR populations could proceed. For an unrelated study, Dr. Kasahara’s laboratory created an siRNA to class I that could effectively knock down class I expression (Haga et al, Transplantation Proc. 38, 3184-3188, 2006). We transduced MDA-MB-231 1833 breast cancer cells with a lentivirus vector. We found the vector to be toxic to 1833 breast cancer cells. Initially we obtained high transduction efficiency (approximately 94%), however, as we passaged the cells more lost their dsRed expression. In 3 weeks more than 75% of the cells lost the marker and by day 30 post-transduction only 14% of them expressed DsRED (data not shown). HLA expression was not analyzed early due to limited cell numbers. A day 39 post-transduction we analyzed one transduced population where we obtained about a 20% reduction in HLA expression compared to the parental cells (data not shown). Experimentally, if we continue to use this vector to knock down HLA class I expression to look at the effects on killing by alloreactive CTL, the experiments should be performed over a time course; we might anticipate better effects would be seen before day 10 such that the down regulation of HLA is maximized. Alternatively, the vector may need to be further concentrated 10 to 50 fold, or we would need to consider repeating the experiments using a different, nontoxic marker vector. The idea would be to sort transduced cells for high, intermediate, and low HLA expression (relative antigen densities via MFI) to test the effects of class I HLA knock down on alloresponsiveness.

**Assessments of transduction of alloCTL with RCR.** By spinoculation we find that 25 to 30% of the alloCTL can be transduced with the RCR containing the GFP marker. We initially reported higher percentages obtained by flow
cytometry, but after further review of the data, the person performing the gating was not doing so properly and the number were overestimates. We are also finding that once the alloCTL are transduced by the RCR, their viability decreases such that the propagation of the RCR is not maintained over the long-term. This may not impact efficacy of the treatment if multiple infusions of alloCTL and RCR, or alloCTL/RCR can be delivered through the implanted cannula. As an alternative approach, however, we obtained a vector EF1a-mCD8-hCD28-IREs-GFP from Carl June, which we considered inserting the telomerase, hTERT, into to immortalize the alloCTL. We have also obtained from Dr. Kasahara other vectors for this study, including Lenti-EF1a-GFP, Lenti-CMV-GFP, Lenti-PGK-GFP, and Lenti-CMV-hTERT-IREs-GFP. These will be concentrated by ultracentrifugation before transducing the alloCTL or alloCTL/RCR.

Key Research Accomplishments In conclusion, these data demonstrate:

• Breast cancer cells transduced with suicide genes and exposed to antiviral agent can exhibit anti-tumor effects.
• HLA knock down in the tumor target cells is possible but technical details regarding type of vector, or concentration of vector used needs to be optimized to obtain reliable results.
• We have transduced a alloCTL with RCR by spinoculation methods and obtain up to 35% transduction before alloCTL start losing viability.
• A variety of vectors have been obtained that may be useful for marking alloCTL/RCR for study or for helping increase the longevity of the alloCTL/RCR in vivo. Should the suicide gene also be incorporated a safety method for stopping their growth in vivo by antiviral therapy would be possible.

AlloCTL in mice with intracranial breast tumors. To examine the therapeutic efficacy of alloCTL against intracranial human breast tumors in immune deficient mice, breast cancer cells were transduced with a lentiviral vector (lenti-fluc-GFP) to express firefly luciferase (fluc). MDA-MB-231 1833 cells expressing high levels of luciferase were injected intracranially into the right hemisphere of female SCID mice at various cell doses. For intravital bioluminescence imaging five days after tumor cell injection luciferin (1.5mg in 100ul) was injected intraperitoneally and light emission was measured 5 minutes later using a NightOwl imaging system (Berthold Technologies, Oak Ridge, TN). Intracranial tumors were detected in all mice injected with fluc-tagged cells (Figure 8). Then alloCTL or vehicle was injected into the same right hemisphere ~1mm posterior or lateral of original tumor injection site and mice were killed 10 hrs later. Brains were frozen and cryosections were stained with H&E. Tumors were seen infiltrating through the brain in clusters essentially mimicking highly disseminated micrometastases. Lymphocytes, presumably human alloCTL were seen tracking through the brain far removed from the instillation site and in proximity with the tumor in hematoxylin and eosin stained sections. Apoptotic tumor cells were also readily visualized. Typical photomicrographs are shown in Figure 9. We have also started developing immunostaining with anti-human antiCD3 to specifically visualize human lymphocytes, i.e. alloCTL or alloCTL/RCR in the mouse brain (Figure 10). Some of the experimental parameters for the animal model have been established. If we continue to find that we can adequately perform these experiments in immuno-deficient mice, we may use them instead of rat brain.

Figure 8. Bioluminescence in the brain of SCID mice injected with fluc-tagged MDA-MB-231-1833 cells five days after intracranial injection. Top animal was injected with 1x105 cells, second animal with 3.3x105 cells and bottom animal with 1x106 cells.
Key Research Accomplishments
In conclusion, these data demonstrate:

- A pilot bioluminescence experiment was performed to assess in vivo imaging of breast cancer cells and trafficking of alloCTL instilled at the tumor site and a few millimeters away from the tumor site.
- Histological examination of breast cancer cell growth in immuno-incompetent rodent brain has begun by hematoxylin and eosin stained sections. As well, we have started working out immunohistochemical techniques for identification of CD3+ alloCTL or alloCTL/RCR in rodent brain.

Reportable Outcomes
Dr. Amy Lin, Postdoctoral Fellow presentation:

Conclusions
We have made adequate progress on the specific aims as outlined in the original proposal and statement of work. We have completed much of the in vitro work and have started looking at the experimental parameters for the animal model established. We will now focus on biodistribution studies and on comparing therapeutic efficacy of human alloCTL, RCR, and alloCTL/RCR against intracranial breast tumors for the remaining funding period of this project.

References
Other Issues:
Dr. Lin worked on this project from September through December 2008, but took a position elsewhere January, 2009. Drs. Kruse and Mueller have searched for an adequate replacement but this proved difficult. Potential employees did not like the uncertainty of the position based upon the financial emergency declared by Sidney Kimmel Cancer Center Board of Trustees. The CEO of SKCC declared Chapter 11 on April 17, 2009. Dr. Mueller also has since relocated her laboratory to the Torrey Pines Institute for Molecular Medicine. Luckily, she is about a 5 minute drive away from Dr. Kruse, who retained her laboratory at the SKCC building; the courts accepted a bid by the Burnham Institute for Medical Research to take over the building and operations as of July 1, 2009. Both Drs. Mueller and Kruse have been in a disruptive environment. Going forward, the infrastructure at the Burnham Institute is very good and their core facilities should enhance the continued work outlined in the proposal.
Immune and Gene Therapies for Breast Cancer Metastatic to the Brain.

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Patients with advanced cancer frequently experience morbidity and mortality due to metastases to brain. Current treatments for brain metastases are ineffective. New therapeutic strategies to treat cancer metastatic to brain are desperately needed. An immunotherapy approach utilizing intratumoral adoptive transfer of alloreactive cytotoxic T lymphocytes (alloCTL) that recognize non-self class I and II human leukocyte antigens (HLA) has shown promise preclinically and clinically for primary malignant brain tumors. Additionally, a gene therapy approach utilizing prodrug therapy following tumor transduction with tumor-selective replication competent retroviral (RCR) vectors carrying a suicide gene also showed promise preclinically in a brain tumor model. We have explored the efficacy of these individual therapies in models of breast cancer metastatic to the brain. We evaluated the individual functionalities of human alloCTL and RCR vectors towards breast cancer cell lines in vitro. We first showed that a panel of human breast cancer cell lines express HLA. We typed the HLA of the human BCM2 BG2 cells that have a propensity to metastasize to the brain and found that they express HLA A24, B35,62 and Cw3,4. Next, we demonstrated that human alloCTL could be generated by mixed tumor lymphocyte reaction, using irradiated BCM2 BG2 cells with upregulated HLA expression, induced by interferon-gamma incubation. Then we demonstrated that alloCTL preparations display cytotoxicity to the relevant target cells in a HLA-restricted manner. With RCR vectors, we showed that those expressing a marker gene were able to infect and replicate in BCM2 BG2 cells and that the RCR vector genome remained stable in these cells. Furthermore, RCR vector expressing a suicide gene were able to infect and eradicate breast cancer cells in the presence of the prodrug. Together, these data suggest that alloCTL are functionally capable of recognizing and injuring breast cancer cells and that suicide gene therapy with prodrug/RCR vectors retain their functional capacity to spread and injure breast cancer cells. Therefore, the efficacy demonstrated in primary brain tumors with each of these modalities may now be extrapolated to breast cancer metastatic to the brain. Furthermore, we will test the individual modalities, then combination of the two, in vivo against single and multiple metastatic breast cancer foci in the brain.