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14. ABSTRACT

The goal of the project is to demonstrate uptake and therapeutic effect of a specific monoclonal antibody, RL6A, by brain metastases of human breast cancer cells in a mouse model. In this report we describe the results from a series of severely immune compromised NSG mice, which had stereotaxically implanted tumor xenografts in the striatum. Treatment with the RL6A antibody (specific for a tumor antigen) or an istotype control did not show differences in survival. However, these mice frequently developed bone metastases in the skull, which is unintended and interferes with the in vivo imaging of tumor growth. Custom breeding of a novel mouse strain, which should be better suited for this project is currently in progress at Jackson Laboratories and availability of mice for experimental use is expected for fall 2015. In the past year we were successful in establishing a method to isolate pure populations of viable human tumor cells from the brain tumors in mouse brain in high yield.

15. SUBJECT TERMS

Experimental Breast Cancer, Brain Metastasis, Tumor Cell Isolation, T-Cell Receptor Mimic Antibody

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Introduction

The increased occurrence of brain metastases in breast cancer patients is a major clinical problem that becomes more relevant with the longer survival times secondary to better therapeutic options now available to treat the primary tumor and metastatic disease in peripheral organs. Unfortunately, current therapeutic options for brain metastases are dismal. The most significant obstacle to progress in the treatment of brain metastasis is the limited penetration of anticancer drugs (small molecule based agents as well as macromolecules like antibody-based drugs) into the CNS tissue. Although tumor vasculature may be leakier than the healthy blood-brain barrier (BBB), drug levels achievable in the vast majority of brain metastases are below drug concentrations in peripheral tumor metastases by almost a log order or more (1). The purpose of the present project is to evaluate a novel approach, in which we exploit the unique property of a particular antibody, dubbed RL6A, to both undergo active transport across the BBB (2) and to have significant antitumor effects (3). RL6A belongs to a novel class of antibodies called T-cell receptor mimics (TCRm), because these antibodies have binding specificities analogous to T-cell receptors. In case of RL6A, this antibody recognizes the peptide YLLPAIVHI (in the following abbreviated YLL-peptide), which is derived from p68 RNA helicase (a tumor antigen), only when presented in the binding groove of human HLA-A2 (4). The project has two specific aims:

Specific Aim #1: Determine the uptake of the TCRm RL6A by breast cancer metastases in brain
Specific Aim #2: Determine the therapeutic effect of RL6A in the brain metastasis model

Body

As discussed in the previous annual reports, the animal model proposed in the original application was a HLA-A2 transgenic mouse strain (Jackson Labs stock #9617) on the NOD scid gamma (NSG) background, i.e. a strain with an almost absent immune system, and the non-transgenic NSG strain. These mice developed tumors in many organs after intracardiac injection of a brain seeking subclone of human MDA-MB-231 breast cancer cells, not just in brain as intended. We have therefore adopted an alternative strategy to conduct the studies proposed in our Statement of Work: (a) stereotaxic injection of tumor cells into the striatum, which should limit tumor growth to the site of inoculation and (b) replacing the strain 9617 mice by custom breeding of a novel HLA-A2 transgenic strain based on the athymic nude mouse background, which has a partial immune system, which may limit metastatic growth in peripheral organs after intracardiac injection of MDA-MB-231BR cells. Corresponding amendments to the animal protocol have been submitted in a timely fashion to ACURO, and approval has been received. Similarly, budget adjustments for the ongoing no-cost extension of this project have been approved recently.

During the past year we performed the following work (with reference to tasks as described in the statement of work):

Tumor model in NSG mice (studies supporting *tasks 3 and 6*):

We used 2 groups of female HLA-A2 transgenic mice on the NSG background (strain 9617, n = 8 per group) and a group of non-transgenic NSG mice (Jackson stock #5557, n = 8) for stereotaxic implantation under isoflurane anesthesia. Using a 33G cannula and a microinfusion pump (2µL over 15 min) we injected 250,000 cells of the luciferase transfected MDA-MB-231BR line into the striatum. Live imaging with the Caliper IVIS was performed every 2-3 days. Treatment with RL6A antibody (one group each of 9617 and 5557 mice) or the isotype control UPC10 (one group of 9617 mice) by i.v. tail vein injection started when the bioluminescence signal over the skull became positive and was repeated every 2nd day until the animal was euthanized. The transgenic mice were randomly assigned to treatment with UPC10 or RL6A, and treatment was performed in a blinded fashion. **Figure 1** shows representative images of

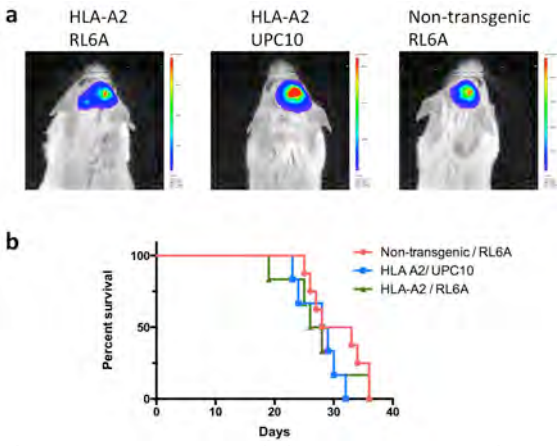

Figure 1. Treatment study in HLA-A2 transgenic and non-transgenic mice. (a) Bioluminescence images shortly before euthanasia in 3 mice. (b) The survival curve showed no significant difference among groups.

one animal in each treatment group. Euthanasia was performed according to the IACUC criteria when the weight loss of an individual animal exceeded 20%, or when the mouse showed signs of significant discomfort (hunched posture, ruffled fur, decreased mobility). There was no difference in survival between treatment groups in this small study (p= 0.38, log-rank Mantel-Cox test, GraphPad Prism 6). After the animals were euthanized, we inspected the skull and brain macroscopically and noticed in many of the animals that tumors had also started growing in the dura and/or parietal skull bone. This explained observations during the IVIS imaging series, where some of the mice developed strong bioluminescence signals at the later time points over the head (e.g. the HLA-A2/UPC10 animal in Fig. 1). On the other hand, these tumors outside the brain tissue prevented quantitative evaluation of brain tumor size based on the in bioluminescence signal. We suspect that even very few tumor cells, which may get scattered along the needle tract during the retraction of the injection cannula may be sufficient to develop tumors over time in the NSG mice. In conclusion, the stereotaxic implantation/treatment study provided an additional argument that NSG mice are unsuitable as a host strain when studying brain tumors of the MDA-MB-231 cell line, even with local inoculation.

Isolation of Human Tumor Xenografts from Mouse Brain (studies supporting *task 5*):

With these experiments we want to establish a technique, which will allow quantitative analyses of tumor cells obtained ex vivo from brain tumors by different methods (e.g. apoptosis markers, protein and gene expression patterns using ELISA, flow cytometry, RT-PCR etc.). Pure human tumor cell populations from xenografts can be isolated by gentle enzymatic/mechanical dissociation of mouse brain tissue using the GentleMacs Octo Dissociator, a brain tumor dissociation kit, a mouse cell depletion kit and the AutoMACS magnetic cell separator (all reagents and instruments from Miltenyi Biotec). We first tested the procedure by spiking a fresh brain sample (50 mg) from a CD1 mouse with a defined number of MDA-MB-231BR cells. Trypan blue staining revealed a recovery of >50% of added tumor cells and >90% viability. Flow cytometry confirmed the viability and retention of the surface marker (HLA-A2). The data are depicted in Fig. 2a. Next we stereotaxically implanted MDA-MB-231BR cells into the brain of an immune compromised mouse and euthanized the animal

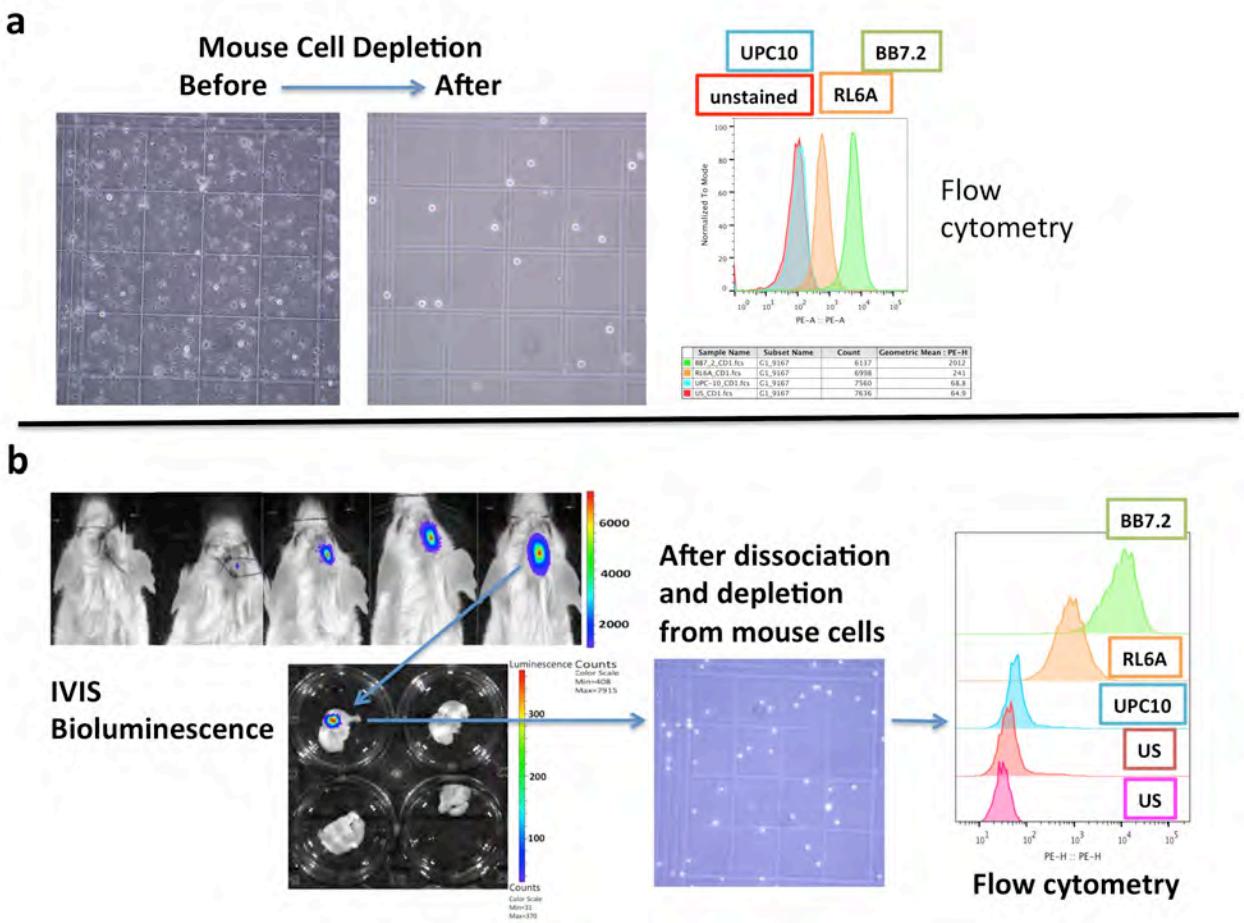

Figure 2. (a) Isolation of MDA-MB-231BR from spiked brain tissue, and (b) isolation from brain tissue carrying the tumor. BB7.2 is a pan-HLA-A2 antibody, RL6A recognizes specifically the YLLPAIVHI-petpideHLA-A2 complex. US = unstained, UPC10 = isotype control for RL6A.

when the tumor was grown to give a substantial bioluminescence signal. Fig 2b a shows the IVIS images of the animal and the brain slice containing the bulk of the tumor. The tumor cell population after mouse cell depletion by negative selection was pure and all cells were viable (see Fig. 2b), and flow cytometry showed staining for HLA-A2 (by BB7.2) and for the YLL-HLA-A2 complex (by RL6A).

Key Research Accomplishments

- We have demonstrated that NSG mice will grow brain tumors after local stereotaxic injection of the brain-seeking subclone MDA-MB-231-BR. However, most of these animals also grow tumors in the skull at the site of injection, which makes it impossible to use IVIS bioluminescence signal to quantify growth of the brain tumor.
- The MDA-MB-231 tumor cells can be isolated with good yield and complete viability from brain tumor xenografts.
- Breeding of a novel nude mouse strain based on B6.Cg-*Foxn1nu*/J carrying HLA-A2 is underway at Jackson Laboratories. The breeding colony has reached the stage where we expect offspring homozygous for the transgene, which would allow in future maintenance of a homozygous colony (provided homozygous animals are breeding). We are currently waiting for genotyping results of pups from crosses of hemizygous males and females.

Reportable Outcomes

- Submission of a manuscript on the work of this project has been delayed by the difficulties with our mouse model.
- An abstract entitled *A TCR mimic antibody crosses the blood-brain barrier and retains binding affinity*, by U. Bickel, R. Bhattacharya, O Hawkins, A. Rawat , J. Weidanz, describing a method contained in the 2014 report has been submitted and accepted for presentation at the *11th International Conference on Cerebral Vascular Biology*, Paris (France) July 6-9, 2015.

Conclusion

With the new transgenic mice expected to become available in sufficient numbers for our planned experimental series by fall of 2015, we anticipate that we can conduct treatment and pharmacokinetic studies in tumor bearing mice as proposed in the recently approved animal protocol / budget revision. Because the required analytical assays (for antibody binding affinity, tumor cell isolation) have been established, we should be able to complete these remaining in vivo studies in a timely manner (by April/May 2016).

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