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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. As described in the previous annual report, both the immune compromised NOD/Scid/Il2Rγ (= NSG), HLA-A2 transgenic mouse strain and the non-transgenic NSG control strain develop metastatic tumors in peripheral organs after intracardiac (left ventricular) administration of tumor cells. We have subsequently explored the use of intracarotid artery administration. However, even though CD1 mice used in pilot intracarotid injection experiments all tolerated unilateral occlusion of the common carotid artery (necessary to avoid bleeding after injection), most of the mice from NSG strains (JAX 5557 and 9617) died during or shortly after the procedure. Therefore, we are currently conducting a series of stereotaxic intracranial injections. As a second alternative we have initiated the custom breeding of novel HLA-A2 transgenic mice on a nude/nude background, which should avoid the problem of peripheral metastases seen with NSG mice.

15. SUBJECT TERMS
Experimental Breast Cancer, Brain Metastasis, Immunoradiometric Assay, 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

Because the RL6A antibody is restricted to human HLA-A2, any animal study aimed at taking advantage of the transport through endothelial cells mediated by the target of RL6A (YLL-peptide / HLA-A2), needs to be performed in HLA-A2 transgenic models, e.g., HLA-A2 transgenic mice. For the present project, a second prerequisite is a state of immune deficiency in mice to facilitate the growth of human breast cancer xenografts (in this project: brain metastases). Based on these considerations, and after screening several commercially available HLA-A2 transgenic strains before the project was started, we decided to use strain NOD.Cg-*Prkdc*^{scid} *Il2r*^{gtm1Wjl} Tg(HLA-A2.1)1Enge/SzJ (Jackson Labs stock #9617), which is bred at Jackson Labs at the level of a "live repository" stock. As a control strain we chose the non-transgenic parent strain NOD.Cg-*Prkdc*^{scid} *Il2r*^{gtm1Wjl} (stock #5557). The latter is also known as NOD-scid-gamma or "NSG", a frequently used strain in preclinical cancer research involving tumor xenograft studies (5). We were aware that the vast majority of breast cancer brain metastases studies, even when using the "brain selective" subclone MDA-MB-231BR, have been conducted in nude (athymic) mice, not in *scid* mice. However, to the best of our knowledge, there was no HLA-A2 transgenic mouse model on the nude background available (either commercially or from academic labs) or even described in the literature. Unfortunately, as we had to realize during the course of our studies, and as described in the first annual report in May 2013, the difference between nude mice (which lack T-cells, but still have a partially functional adaptive and innate immune system) and NSG mice (near complete absence of both adaptive and innate immune response) appears to be critical with regards to where tumors grow after systemic injection of cancer cells.

Body

In the second year of this grant we have 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*)
In order to avoid tumors in peripheral organs, as encountered with the intracardiac administration (where injection of MDA-MB-231BR invariably results in wide spread tumor growth in different organs) we evaluated a modification of the injection technique by directly injecting cells into the internal carotid artery. This should significantly increase the fraction of cells reaching the cerebral circulation. For comparison, cerebral blood flow in mice constitutes only about 5% of cardiac output. Carotid artery injection in mice is technically challenging, but our group has considerable experience with a related

technique, the middle cerebral artery occlusion (MCAO) technique, used in other projects. In this method a filament is advanced into the internal carotid artery for the induction of stroke. We have also consulted with Dr. Dihua Yu's group (MD Anderson Cancer Center), who utilizes the intracarotid injection method of MDA-MB-231 cells(6). After receiving approval by the animal care committee for changing the injection procedure, we first tested intracarotid injections in a series of normal mice (CD1 strain) with physiologic saline. Under inhalational anesthesia we surgically exposed the carotid artery at the neck and injected with a 30G cannula. The technique involves occlusion of the common carotid artery on the side of injection to avoid major bleeding following withdrawal of the cannula. Most strains of mice have a well-developed Circle of Willis, and therefore sufficient compensatory blood supply to brain coming from the contralateral side and from the vertebral arteries. Accordingly, this technique worked well in the pilot series with CD1 mice. Unfortunately, the NSG mice (both non-transgenic strain 5557 and HLA-A2 transgenic 9617 mice) turned out to be much more sensitive to acute closure of one common carotid artery. All mice died either during the procedure or shortly after waking up from anesthesia (total n = 12). We have not analyzed the anatomical features of the NSG strain regarding the Circle of Willis, but we assume that it is insufficiently developed in this strain to tolerate the acute unilateral closure of a carotid artery.

In an attempt to avoid complete closure of one common carotid artery, we tested in a few animals a variation, the retrograde catheterization of the external carotid artery. This allows to tie down only the external carotid artery after injection of cells, and to reopen the common carotid and internal carotid artery at the end of the procedure. The modified approach is technically feasible, yet difficult and time consuming in the mouse. One animal of a small pilot series (n = 4) received an injection of 50,000 MDA-MB-231BR by this injection route. Unexpectedly, when the animal was sacrificed 6 weeks later and the organs processed for H&E staining, we found histological evidence of metastatic tumors in liver and lung. A single, small tumor was also detected in brain, and another, larger tumor had developed in a subdural location. Based on these findings, we have abandoned any further efforts to generate brain tumors in NSG mice by systemic vascular injections. Instead, we will use these mice for intracranial tumor implantation by stereotaxic injection into the striatum. Such experiments are currently in progress.

After discussing the options with breeding experts and immunologists at Jackson Laboratories, we have also initiated the custom breeding of HLA-A2 transgenic mice on the C57/B6 nude background (Jackson strain B6.Cg-*Foxn1nu*/J, stock #0819). We are expecting a first shipment of mice in June 2014.

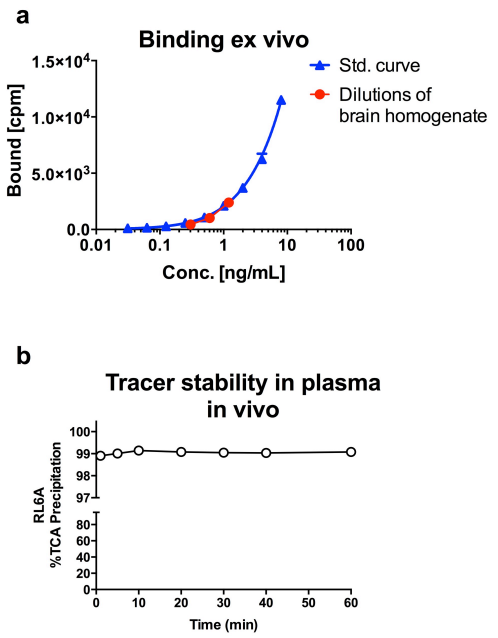

Figure 1. a) Binding curve of ^{125}I-RL6A tracer. The blue curve shows bound tracer as quantified by gamma counting after incubation of increasing concentrations of ^{125}I-RL6A for 2h and washing (3x with PBS buffer) in wells previously plated with the antigen (YLL-peptide HLA-A2). The binding data in red circles show three dilutions of supernatant from brain homogenate obtained 1h after i.v. bolus injection of ^{125}I-RL6A to a HLA-A2 transgenic mouse. **b**) Blood samples drawn from the same animal at different time points were centrifuged and plasma was subject to precipitation with trichloroacetic acid (TCA). The fraction of TCA precipitable tracer was close to 100% at all time points, showing stability of the ^{125}I-RL6A tracer in the circulation.

Demonstrate integrity of TCRm in brain tissue following i.v. injection *(task 4)*

We have accomplished a significant part of this task. In particular we were interested in demonstrating that the antibody maintains its binding affinity after transcytosis through brain vascular endothelial cells. HLA-A2 transgenic mice (strain 9617) received 20 µCi of ^{125}I-labeled RL6A by i.v. injection into the jugular vein (under isoflurane anesthesia). After 60 min the animals were euthanized, vascular content was washed out by perfusion through the left cardiac ventricle with PBS, and the brain removed. ^{125}I-RL6A binding to its target antigen

was then probed using the sensitive immunoradiometric assay (IRMA) as described in the previous annual report. To ensure release of antibody from specific binding sites in tissue, we have homogenized the brain tissue in buffer solution at pH 3. Under these acidic conditions the target of RL6A (YLL-peptide-HLA-A2 complex) is unstable and disintegrates into its 3 components (MHC class I heavy chain, β_2-microglobulin, YLL-peptide), releasing any potentially bound RL6A antibody. Following high-speed centrifugation, the supernatant of the homogenate was neutralized to pH 7.4, and the binding of tracer was probed in the IRMA at 3 dilution steps. Comparison of the binding and dilution steps with a standard curve generated by spiking naïve brain tissue homogenate with ^{125}I-RL6A tracer resulted in near perfect overlay of the curves (see Fig. 1a). This indicates that the binding affinity of the ^{125}I-RL6A tracer was retained tracer after intravenous injection and brain uptake. In addition we could show that the tracer was stable in blood circulation for at least 1 hour, as determined by TCA precipitation of plasma samples (see Fig. 1b) drawn from an arterial catheter over this period.

Flow cytometry of primary endothelial cells isolated from mouse brain.

We proposed to determine the fraction of dead cells / induction of apoptosis in brain-derived endothelial cells (and brain tumor cells) after treatment of mice with RL6A or control antibodies by flow cytometry (*task 6c*). Towards this goal we are establishing flow cytometric assays. We want to be able to stain simultaneously (in the same sample) for live/dead cells (e.g., using the Zombie dye, Biolegend), for a suitable marker of endothelial cells, such as PECAM-1, and for other parameters of choice (e.g. for an inflammatory marker like VCAM-1 or for the expression level of YLL-peptide-HLA-A2). Figure 2 shows an example of a multicolor analysis. In this experiment PECAM positive cells isolated from the brain of HLA-A2 transgenic mice were simultaneously stained using an antibody to VCAM-1 (vascular endothelial cell adhesion molecule) and with RL6A. This type of multicolor flow analysis requires a well-designed protocol and the use of appropriate controls, as depicted in figure. We are currently expanding on these results obtained with the endothelial cells by preparing single cell isolations of MDA-MB-231 tumor cells from brain homogenates, using a tumor cell isolation protocol (GentleMACS Octo dissociator, Miltenyi), as indicated in the original grant proposal.

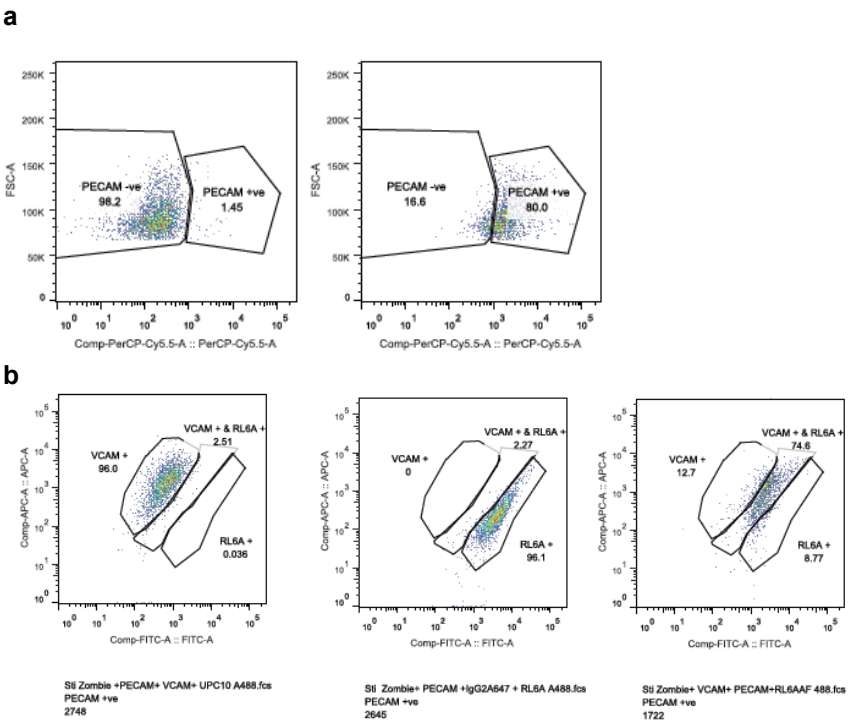

Figure 2. A population of PECAM positive endothelial cells from mouse brain (right panel in **a**) was gated and further analyzed for VCAM-1 and RL6A staining as shown in (**b):** Dual positive (VCAM+/RL6A+) cells stained with VCAM-AlexaFluor647 antibody and RL6A-AlexaFluor488 are shown in the right panel. The left panel shows VCAM positive cells when RL6A is replaced by isotype control, UPC10-AlexaFluor488. The center panel shows RL6A positive cells when VCAM-1 antibody is replaced by the corresponding isotype control, IgG2a-AlexaFluor647.

Key Research Accomplishments

- We have demonstrated that NSG mice will grow breast cancer metastases in multiple organs following either intracardiac or intracarotid artery injection of the brain-seeking subclone MDA-MB-231-BR. We also found that NSG mice do not tolerate acute occlusion of a carotid artery.
- The RL6A antibody is stable in the circulation after i.v. injection and it appears to retain its binding affinity after uptake across the blood-brain barrier.
- A flow cytometry protocol with brain derived endothelial cells isolated from mouse brain has been established, which allowed multicolor analysis of various parameters. This protocol can be adapted to other parameters of interest.

Reportable Outcomes

We are currently compiling the results obtained to date for submission of a manuscript (planned submission in July).

Conclusion

The finding that NSG mice are an unsuitable for generation of breast cancer brain metastases by systemic vascular injection leaves us with two alternatives: Stereotaxic implantation (currently underway) and generation of a novel HLA-A2 transgenic mouse strain based on the nude background (custom breeding is performed at Jackson Labs). We expect that we can conduct intracardiac injection studies in these new mice according to the original plan.

The binding studies with RL6A antibody obtained from brain homogenate after intravenous injection showed integrity of the antibody. This supports our expectation that the RL6A antibody can target metastatic tumors expressing its cognate peptide-HLA complex.

The flow cytometry experiments conducted to date form the basis of analysis of endothelial cells and tumor cells from mice with tumor xenografts.

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