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14. ABSTRACT The central hypothesis of this Idea Award is that a combination of nutritional and immunological treatments may work together to stimulate the body's natural immune defenses against breast cancer. We are testing treatment with 3 agents, retinoic acid (RA), a metabolite of the essential nutrient vitamin A, with immune stimulation using alpha-galactosylceramide (α-GalCer), a synthetic lipid known to alter immune function and to display antitumorigenic activity in vivo; and poly-I:C (PIC), an inducer of type I and type II interferons (IFN). Our research has shown that RA induces CD1d expression and, as a consequence, iNKT cell activation is altered. Research in year 1 has shown that RA potentiates α-GalCer-induced mouse spleen cell proliferation, and alters the balance of NKT1 to NKT2 cytokines by reducing the former type and increasing the latter type. This was also shown by testing the intracellular cytokine mRNA expression in NKT cells (NK1.1-positive CD3-positive spleen cells) detected by flow cytometry. Our studies showed that B cells play a significant role as CD1d expressing cells. B cells are antigen-presenting cells, and thus this cell population should be further considered in the hypothesis we are testing					
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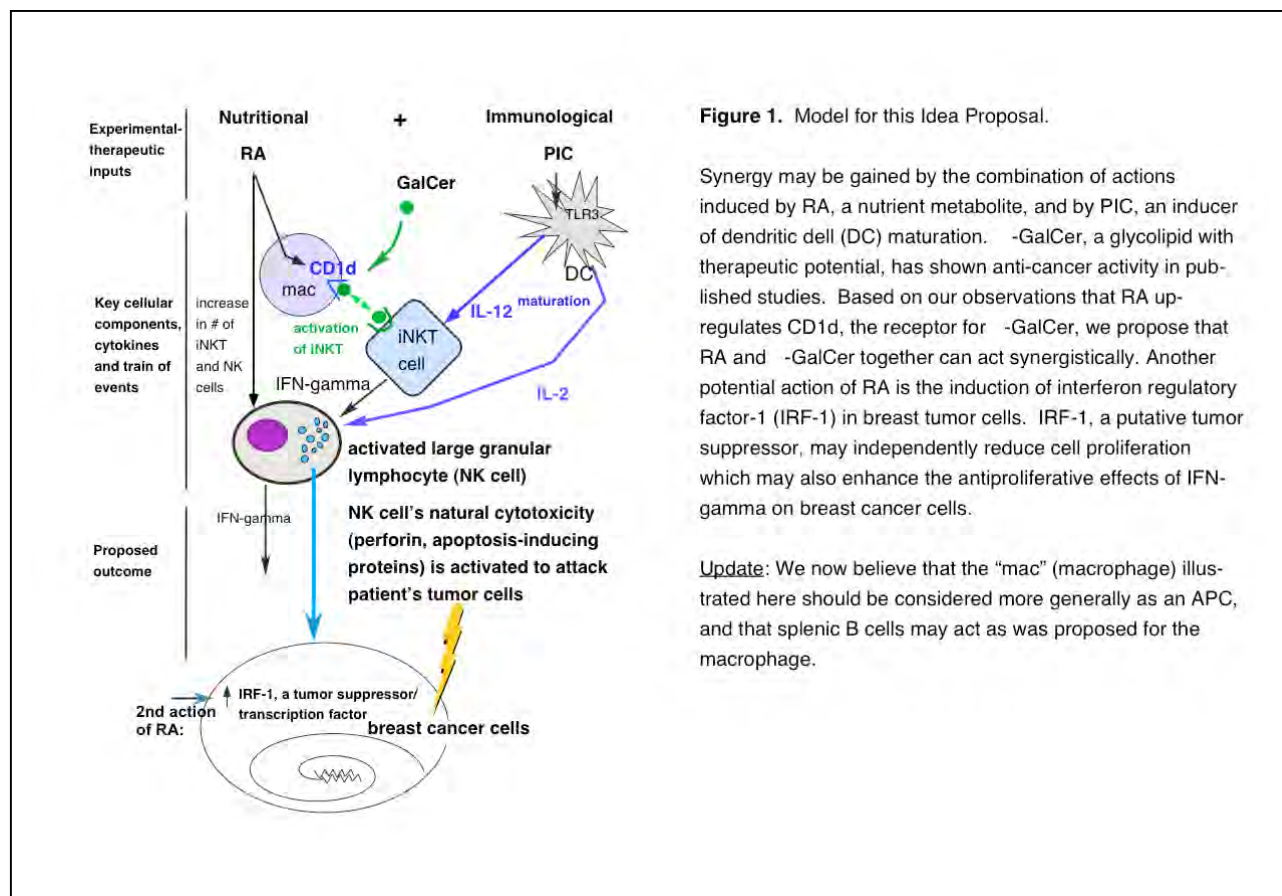
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Introduction

This is a year 1 annual report for the project: **A combined nutritional and immunological intervention to activate natural cytotoxicity against breast cancer cells in vitro and in vivo.**

The central hypothesis of this Idea Award is that a combination of nutritional and immunological treatments may work together to stimulate the body's natural immune defenses against breast cancer. The grant proposal described combining treatment with 3 agents, retinoic acid (RA), a metabolite of the essential nutrient vitamin A, with immune stimulation using alpha-galactosylceramide (α -GalCer), a synthetic lipid known to alter immune function and to display antitumorigenic activity in vivo [1]; and poly-I:C (PIC), a known inducer of type I and type II interferons (IFN). We hypothesized that RA would result in the increased expression of CD1d. CD1d is a major histocompatibility complex (MHC) class I-related molecule that is expressed by several types of cells throughout the body, particularly by antigen-presenting cells (APC), such as dendritic cells (DC). CD1d is directly involved in the stimulation of lymphocytes known as natural killer T cells (referred to as NKT cells, invariant NKT cells, CD1d-dependent NKT cells, or V β 8-expressing NKT cells [2,3]. Since the binding of α -GalCer to CD1d leads to the activation of these NKT cells, and since PIC could potentially further augment the production of cytokines by DC or other cells, including the release of IFNs that enhance innate antitumor immunity, we hypothesized that this triple combination – RA + α -GalCer + PIC – could be effective as a breast cancer treatment. The objective of our award is to test this idea in a mouse model, using adult female Balb/C mice and 4T1 syngenic breast tumor cells as a model [4]. Our hypothesis is based on 1) the work of others showing that α -GalCer, a ligand for CD1d, has potent antitumor effects against the growth of melanoma cells, lung cancer cells [1] and other tumors in several animal models, and 2) our own research showing that RA can induce the expression of CD1d on human and murine monocytic cells [5]. A depiction of our hypothesis is shown in [Figure 1](#). Essentially, we want to test whether treatment with RA, by upregulating CD1d, can enhance a train of event by which α -GalCer, after binding to CD1d, may increase production of IFNs, and this may activate natural killer (NK) cells to fight the tumor cells. We described both in vitro and in vivo studies. A possible advantage, which is unknown at this time, is that the initial treatment of the cancer patient's own immune cells (APCs such as DC) could be done outside the body, using autologous blood cells collected from the patient and activated in culture, and then reintroduced sterilely after treatment. This ex vivo treatment might minimize the exposure of the patient to drugs (RA, α -GalCer) that if given systemically could result in unwanted side effects. Nonetheless, we proposed to test treatments both in vitro with isolated cells and in vivo, by administering RA and α -GalCer to intact mice. Overall, there are several new concepts to be tested in this project.



In year 1, we both achieved some successes and realized some frustrations in our initial studies in this project. It is the purpose of this annual report to explain both. Our main successes were first, in initiating the project in a timely manner and being able to show that RA is an effective inducer of CD1d, and hence of α -GalCer stimulated immune responses. We also obtained preliminary data on cytokine production by α -GalCer-stimulated lymphocytes in culture, and showed that RA modulates the profile of Th1/Th2 (NKT1/NKT2) cytokines. Our frustrations were, first, that the nuclear magnetic resonance (MRI) imaging method we had hoped to use for imaging tumor development in mice was no more sensitive than standard palpation, due to limited tissue density differences between the tumor itself (4T1 tumor cells) and surrounding normal mammary gland tissue in Balb/C mice. This was disappointing, but the imaging scans turned out to require a very long procedure, and we would not have been able to image many mice due to time and costs, even the procedure had worked well. We realized that the less technologically advanced but more tedious methods of palpation, measuring tumors and studying them histologically would be better for this project. Secondly, we found it difficult to obtain bone-marrow derived DC after ex vivo culture in a condition satisfactory for re-injection into recipient mice. As explained below, the DC derived from bone marrow cultures were very adherent to plastic dishes and it was difficult to release them in good condition for analysis and use. After several attempts, we pursued an alternative approach using spleen cells as a source of monocytes. We characterized all of the spleen cells and in doing this we made a serendipitous finding, namely that B cells are the major spleen cell population expressing of CD1d. Thus, we believe we should be able to modify our plans for years 2 and 3 to focus more on B cells as initiators of the CD1d-activated train of events described above. A possible advantage to this modified approach is that B-cells, which are professional APCs, could play additional roles in anti-tumor immunity through antibody production, which we had not originally proposed.

BODY

This section of the report describes our Statement of Work (SOW) and explains how we have proceeded and adjusted our plans according to the data we have obtained.

Our proposal listed 8 tasks and 3 milestones during the first 12-13 months of the project. This was somewhat ambitious. We accomplished some of these, modified others, and postponed some.

Task 1: Done.

We first prepared an IACUC application, which was approved within the time indicated (1-2 months). This approval followed our original research plan exactly, including use of Balb/C mice for our studies, the treatments indicated, and the use of MRI in vivo in laboratory animals.

Task 2: Done.

Inventory supplies and order necessary materials to begin.

We obtained necessary materials. To detect NKT cells, we were able to obtain CD1d-Streptavidin tetramers, APC-labeled for detection, through the NIH-sponsored Tetramer Facility located at Emory University. This was both cost saving and effective. Laboratory workers (Qiuyan Chen, Katherine Restori and myself) completed and/or updated Penn State worker protection courses (Radiation Safety; Animal Care, Animal Worker Questionnaire).

Task 3 (aim 1-A, Months 3-4): Beginning aim 1 – begun and modified.

We had planned to start immediately with studies in which the cells of mice treated in vivo would be studied ex vivo, to test whether IFN-gamma production is necessary in our model. We decided to begin with WT mice only, due to the expense of IFN- γ knockout mice, and to focus first on testing the MRI methodology and our ability to induce and recover activated DC, prepared ex vivo from bone marrow cells.

DC: We obtained murine bone marrow cells in good yield without difficulty. We cultured them using the proposed methods in which the growth and differentiation factors GM-CSF and IL-4 are added to induce DC expansion, followed by addition of TNF α to induce the maturation of the DC. During this culture period of ~9 days, some of the cells were treated with RA, and others were ethanol as a vehicle control. We planned to pulse the DC with α -GalCer, or the inactive isomer β -GalCer as a placebo control, and then to introduce the DC into recipient mice. We carried out this experiment. Two problems surfaced: first, the DC became highly adherent to the plastic culture dishes. Recovering the cells for characterization was very difficult, although we could partially characterize them as adherent cells and by morphology. Thus, the problem was not in developing the activated DC but in recovering them for injection into recipient mice. Even scraping the cells was ineffective, and/or too damaging to them. At the Era of Hope meeting in Baltimore in June 2008, I talked with other investigators and found that our problem was not unique; some researchers suggested culturing in siliconized glassware to reduce adhesion, or culturing the cells on beads and injecting the entire cells-on-beads mixture.

MRI: As noted above, we had proposed to use a new instrument at Penn State to image the tumor cells in vivo, a procedure that we thought would allow for multiple measurements over time. The Small Animal MRI facility had just opened and the methodology had not been applied previously for the purpose of detecting breast tumors. Using several mice injected with 4T1 breast cancer tumor cells (obtained from Dr. Danny Welch, University of Alabama, Birmingham), we conducted MRI one week, and again 10 days and 14 days after tumor cell

inoculation into the inguinal fat pads of Balb/C mice. Initially, we could not visualize the tumor tissue by MRI, and even the distinction of major visceral organs was not as clear as we had hoped. The mice had to be anesthetized with isoflurane for a long period, up to an hour, to obtain good images, and the duration of the anesthesia, which varied among mice, was a concern to us. After the tumors had grown to a palpable size, then they also were detectable by MRI. But since the purpose of using the MRI was to obtain early and sequential measures, we decided that the standard procedure of palpation and tumor measurement, using an accurate digital caliper that we purchased, would be just as effective. Thus, the MRI part of the study was not as promising as we had hoped it would be, although it helped to establish a limitation of the method for other investigators.

Task 4, CD1d expression and iNKT cell analysis. Done.

We analyzed CD1d directly with an FITC-labeled monoclonal antibody, by flow cytometry, and iNKT cells by similar procedures using CD1d tetramer-streptavidin, which binds to the specific T-cell receptor present on the NKT cells, for detection. Four treatment groups were tested: 1) vehicle (control), 2) RA, 3) α -GalCer and 4) RA plus α -GalCer combined. We conducted this study and showed that RA and α -GalCer result in upregulation of CD1d, and increased numbers of iNKT cells.

Tasks 5 and 6, cytotoxicity assays. Modified.

Due to the difficulties described for recovery of DC in task 3, we decided to postpone this task, and focus instead on testing other sources of DC, or other CD1d-expressing cells. We first tested Balb/C mouse spleen cells, thinking that splenic monocytes would express CD1d and might be a reasonable substitute for bone marrow-derived DCs. Additionally, we have had considerable experience preparing and handling spleen cells and expected they would not pose the adherence problems we had encountered with bone marrow-derived DCs. We first characterized the total spleen cell population using multi-color flow cytometry and a panel of monoclonal antibodies for B cells (CD19 marker), monocytes (CD11b) and DC (CD11c), after excluding T cells. Initially to our surprise, the results showed that the majority of the CD1d in spleen is present on B cells. The monocytic population had some, but a relatively small percentage, of the total CD1d in spleen. One published report has indicated a role of B cells in CD1d-antigen presentation to NTK cells [6]. Additionally, B cells are known to be professional APCs, so this finding is not unreasonable, although we had thought monocytes would be the major cell type expressing CD1d. Based on this, we have modified our plan to concentrate more on B cells as CD1d-expressing cells, and to test their ability to activate NKT cells, and thus to function in the nutritional-immunological defense against breast cancer cells.

Milestone 2: Modified.

Due to the results above, we have not reached milestone 2 exactly as planned. However, we have obtained some unexpected novel data on the activation of B cells by RA and α -GalCer, and are close to preparing a manuscript on this topic. Thus, milestone 2 will be reached in slightly modified form. We decided to add an additional (not in the original proposal) experiment comparing two strains of mice, Balb/C as proposed, and C57Bl/6, since these two strains can differ in their cytokine and NK cell responses and we want to be very sure that the results we have produced in Balb/C mice can be reproduced in a different strain. At the completion of this experiment, which is currently in progress, we will begin preparation of a manuscript based on these results.

Task 7--Cytokine assays. Modified slightly.

Our plan was to determine the production of cytokines, by cultured NK and iNKT cells. NKT cells are unusual in their ability to produce both type 1 and type 2 cytokines, very rapidly and at a high level. We modified this task slightly to focus more on NKT1/NKT2 balance, by

measuring IFN-gamma and IL-4, signatures of type 1 and type 2 cytokine responses, respectively. As analyzed by ELISA and also by real-time PCR, α -GalCer strongly stimulated both IFN-gamma and IL-4 production. Interestingly, the addition of RA resulted in a reduction of the induced IFN-g response (still elevated over control, but ~50% of the level with α -GalCer alone), while at the same time RA significantly increased the production of IL-4. Overall, the ratio of IL-4 secretion to IFN-gamma secretion was increased 5-fold in the presence of RA (Fig. 2C). We have yet to determine the effect of this change on the activation of NK cells, in the model above.

Task 8 (Months 12-13): In progress.

We are in the process of completing analysis of all data by ANOVA, and will include the new experiment with the two mouse strains, as described above. We are organizing the data for publication and writing a manuscript. We presented some of these data as an abstract and poster (P51-9, Immune-based Therapies 2, June 27, 2008, at the Era of Hope meeting). At the conclusion of task 8, we will have learned RA, α -GalCer and their combination affect mouse splenocyte populations, iNKT activation, and the lytic potential of NK cells.

Milestone 3: Manuscript submission on aim 1, on in vitro cell activation. Expected to be completed by October 1, 2008.

Key research accomplishments

Our research has shown that RA is an inducer of CD1d expression and that, as a consequence, iNKT cell activation is altered. As illustrated in [Figure 2](#), we obtained results that 1) RA potentiates α -GalCer-induced mouse spleen cell proliferation (panel A), and 2) RA alters the balance of NKT1 to NKT2 cytokines (mRNA, panel B; protein secretion, panel C), reducing the former type and increasing the latter type. This was also shown by testing the intracellular cytokine mRNA expression in NKT cells (NK1.1-positive CD3-positive spleen cells) detected by flow cytometry.

Figure 2.

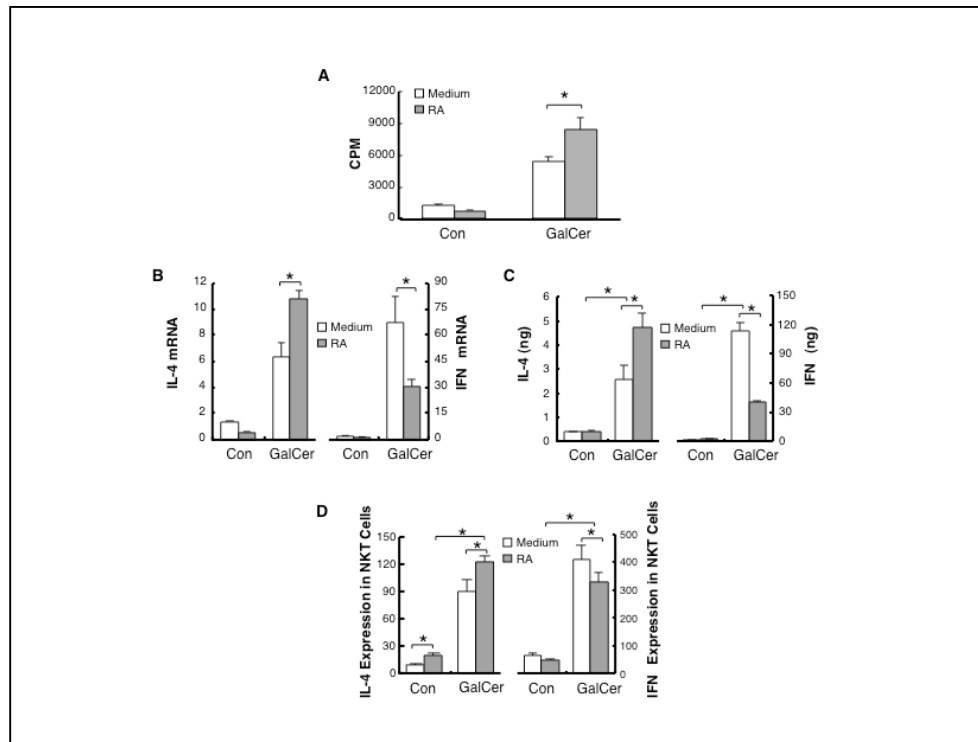


Figure 2. RA regulates CD1d/ α -GalCer-mediated NKT cell functions. Mouse spleen cells were cultured in the presence and absence of α -GalCer and/or RA for up to 3 days. Cell proliferation was determined each day by ^3H -thymidine incorporation, or cells were harvested for RNA isolation and cell staining and cell culture supernatant was collected for ELISA to determine cytokine production. **A.** Cell proliferation was induced by α -GalCer and enhanced by RA at 72 h. **B and C.** RA regulated α -GalCer-induced IL-4 and IFN γ mRNA (real-time RT-PCR, panel B) and protein secretion (ELISA using cell supernatant, panel C) at day 3. **D,** RA regulated the α -GalCer-induced expression of intracellular cytokines (IL-4 and IFN γ) determined by flow cytometry on day 3.

Reportable outcomes

We have not yet reported our results, except in abstract form. As noted above, we are close to completing studies for a first manuscript. A copy of our Era of Hope abstract follows:

Retinoic acid and alpha-galactosylceramide, a ligand for CD1d on antigen-presenting cells, differentially regulate the production of immunoregulatory cytokines by cultured dendritic cells and splenocytes.

A. Catharine Ross, Qiuyan Chen, and Katherine H. Restori
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Background and objectives: Activation of the natural immune system is promising as a way to inhibit tumor growth. Dendritic cells (DC), natural killer (NK) cells, and a specialized subset of T cells known as iNKT cells are among the cell types likely to inhibit tumor growth in vivo. Based on previous results, we postulated that a combination of retinoic acid (RA), an agent that often inhibits cell growth and induces cell differentiation, and which can induce the expression of CD1d by antigen-presenting cells, may augment the activation of iNKT cells in the presence of α -galactosyl-ceramide (α -GalCer). In animal models, α GalCer has shown encouraging results against several types of cancer, but to our knowledge it has not yet been tested in combination with RA or cytokine-inducing agents such as poly-I:C, a strong inducer of interferons. We thus have proposed that a triple nutritional-immunological combination of RA, α -GalCer and poly-I:C could be effective for breast cancer prevention, based on 1) the ability of RA to induce CD1d, 2) of α -GalCer to bind to CD1d on antigen-presenting cells (dendritic cells, DC, or macrophages) and activate iNKT cells, and 3) of poly-I:C to stimulate the production of cytokines, especially IFNs, which also activate NK cells. We plan to test the basic concept that these agents may synergistically inhibit tumor growth, in cultured cells and in mice in vivo. In the first 6-month period of our award, our objective was to test whether α GalCer combined with RA can regulate the proliferation of cultured DC and splenocytes, and their production of immunoregulatory cytokines.

Methods

DC were prepared from the bone marrow of the femur and tibia of adult (> 8 wk) female Balb/c or C57BL/6 mice. The cytokine GM-CSF was added to the cultures in complete media every 3

days for total of 9 days to induce growth of immature DC. On day 9, $\text{TNF}\alpha$ (5 ng/ml) was added to induce DC differentiation. Splenic mononuclear cells were treated with RA $\pm$ α GalCer for 24 hr. To monitor NKT cell proliferation, differentiated DC or isolated splenocytes in 96-well plates were treated with RA (20 nM) or α -GalCer (100 nM) for 24 h. The inactive anomer, β -GalCer (100 nM), was used as control. Two NKT cell lines, as potential responders to the presentation of α -GalCer bound to CD1d, were then added to the DCs or splenocytes for 48 h. For the last 4 hr, 3H-thymidine was added to monitor cell proliferation.

Results to date

In our preliminary studies:

1. RA significantly reduced the rapid proliferation of the two NKT tumor cell lines, DN32.2 and TCB11. Proliferation was also decreased in co-cultures of NKT cells with DC, but not with spleen cells.
2. Spleen cells produced and secreted IL-4 and IFN γ only when cultured with α GalCer. The presence of the NKT tumor cells, especially TCB11, further increased spleen cell cytokine production, although these cells alone produced neither cytokine.
3. The production of IL-4 and IFN-gamma by spleen cells was differentially regulated by RA, as RA increased the output of IL-4 when α -GalCer-activated spleen cells were co-cultured with NKT cells, but at the same time RA reduced the output of IFN-gamma.

Conclusions: The growth-inhibitory effects of RA against the NKT tumor cells is encouraging, but further in vivo studies are needed. α -GalCer markedly induced IFN-gamma production by splenocytes. The attenuation by RA needs to be further evaluated, especially with PIC included in the triple stimulation model.

(Penn State IACUC #24967; funding from U.S. Army Medical Research Award, Number W81XWH-07-1-0478.)

Conclusion

Year 1 has resulted in an effective start on this project. We have made decisions based on the data that have taken us slightly away from the original plan of work. However, the use of DC was not integral to the hypothesis, and any stimulation of APC functions by induction of CD1d, whether on B cells, monocytes, or other cells, could be consistent with the model shown in Figure 1. We have not yet incorporated PIC into our studies, as that was a goal of aims 2 and 3, which were not planned for year 1. We remain on track. We find the project exciting and not duplicative of any other research of which we are aware.

For year 2, we plan to continue by picking up the part of the study testing the essentiality of IFN-gamma as a central regulator of cytotoxicity in this model. As proposed, studies with RA and α -GalCer will be conducted in parallel using cells from wild type and IFN-gamma KO mice.

Also in year 2 we will begin and conduct most of Aim 2. We will test in vivo the ability of transferred cells (which now may be splenic cells), treated in vitro with RA to induce CD1d and α -GalCer to bind to CD1d, to stimulate iNKT cells and inhibit the growth and metastasis of 4T1 murine breast carcinoma cells in syngeneic Balb/C mice. Tumor progression will be monitored after 10-14 days using standard tumor detection measurement techniques, and confirmed by histology at the end of the study.

Looking ahead to year 3, we plan to conduct aim 3, designed to determine whether adding a continuous in vivo treatment, including PIC, will be more effective than the simple ex vivo treatment with RA and α -GalCer tested in aim 2, and whether IRF-1 protein [7] is differentially expressed in tumor and adjacent normal tissue.

References

1. Toura I, Kawana T, Akutsu Y, Nakayama T, Ochiai T, Taniguci M. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide. *J Immunol* 1999; 163:2387-2391.
2. Godfrey DI, Kronenberg M. Going both ways: immune regulation via CD1d-dependent NKT cells. *J Clin Invest* 2004;114:1379-1388.
3. Kronenberg M, Gapin L. The unconventional lifestyle of NKT cells. *Nat Rev Immunol* 2002;2:557-568.
4. Samant RS, Debies MT, Hurst DR, Moore BP, Shevde LA, Welch DR. Suppression of murine mammary carcinoma metastasis by the murine ortholog of breast cancer metastasis suppressor 1 (Brms1). *Cancer Lett* 2006;235:260-5.
5. Chen Q, Ross AC. Retinoic acid regulates CD1d gene expression at the transcriptional level in human and rodent monocytic cells. *Exp Biol Med (Maywood)*. 2007;232:488-94.
6. Galli G, Nuti S, Ravarini S, Galli-Stampino L, De Lall C, Csorati G, Dellabona P, Abrignini S. De1d-restricted help to B clls by human invariant natural killer T lymphocytes. *J. Exp. Med.* 2003; 197: 1051-7.
7. Luo XM, Ross AC. Retinoic acid exerts dual regulatory actions on the expression and nuclear localization of interferon regulatory factor-1. *Exp Biol Med (Maywood)* 2006;231:619-31.

Appendices

None.