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Cytotoxicity Against Breast Cancer Cells in Vitro and In Vivo

PRINCIPAL INVESTIGATOR: A. Catharine Ross, Ph.D.

CONTRACTING ORGANIZATION: Pennsylvania State University
University Park, PA 16802

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14. ABSTRACT The hypothesis of this Idea Award is that a combination of nutritional and immunological treatments may be effective in stimulating the body's natural immune defenses against breast cancer. The project is examining whether treatment of dendritic cells (DC) with retinoic acid (RA), a metabolite of vitamin A shown to modulate the differentiation and/or activation of several types of immune cells, followed by pulsing the DC with α -galactosylceramide (α -GalCer), a synthetic lipid known to alter immune function and to exert antitumorigenic activity, will reduce the growth and/or stimulate natural immunity against 4T1 tumors in adult female Balb/C mice. 4T1 is a syngenic breast tumor cell that does not evoke an immune response. However, natural immunity might help to fight the tumor. Results in the past year have shown that RA + α -GalCer-treated (pulsed) DCs, administered to mice with 4T1 cells, alter immune cell populations, and reduce tumor size. While the tumor alone alters immune cell populations, DCs restore a more normal pattern. The effect on RA and α -GalCer is not directly on the growth of the 4T1 cells. Our studies with DCs will be repeated in year 3 with modifications to increase the sample size and improve the timing of tumor assessment. The results to date support the idea that a nutritional and immunological combination treatment of host DCs could be effective in reducing the growth of breast tumors.					
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Table of Contents

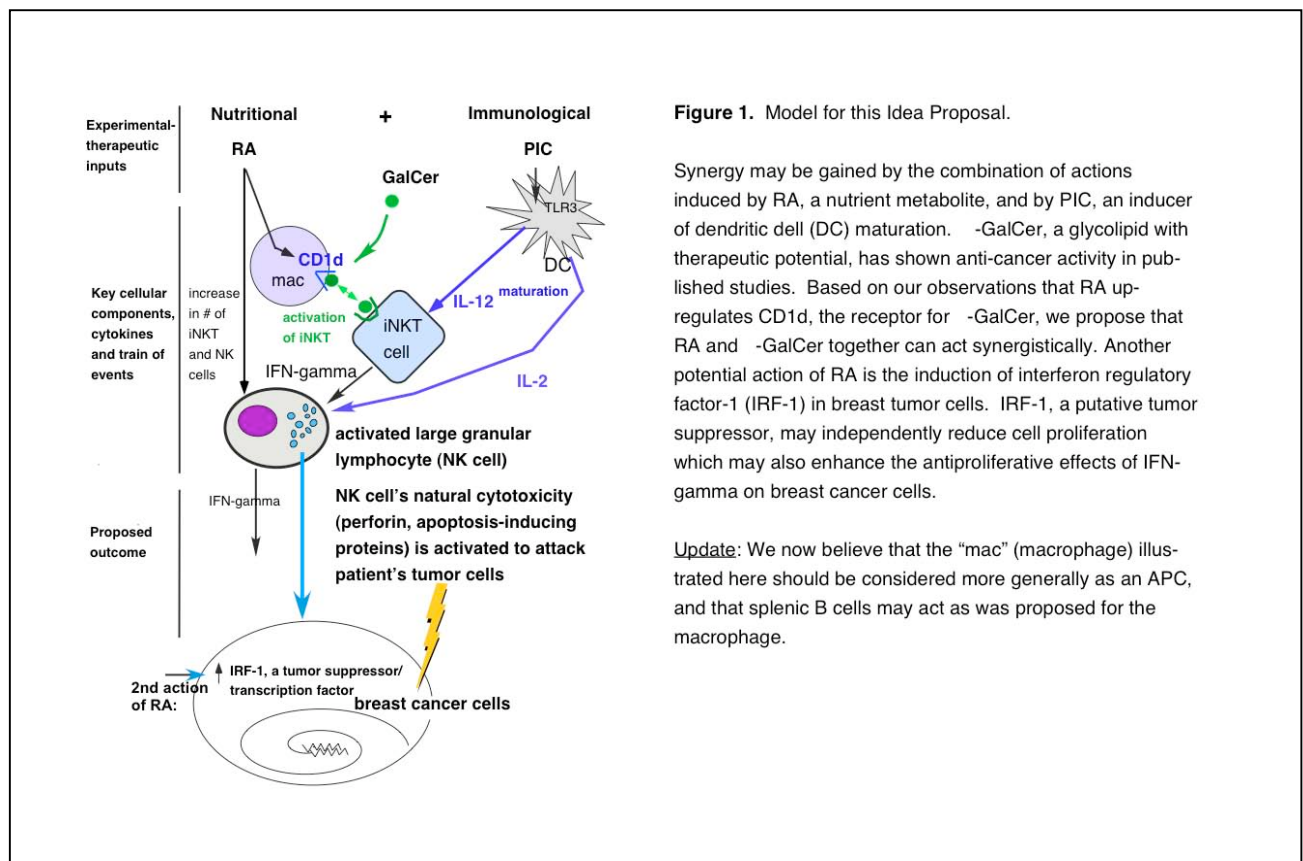
	<u>Page</u>
Introduction.....	5
Body.....	7
Key Research Accomplishments.....	6
Plan for year 3	15
Reportable Outcomes.....	17
Conclusion.....	19
References.....	20

Body

Introduction

This is a year 2 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 conceptual model for our studies is shown in [Figure 1](#). In our original grant proposal we described the potential immune stimulation, against breast tumor cells, that we hypothesized could be achieved by combining 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). We also included poly-I:C, known to be inducer of type I and type II interferons (IFN) as another possible means of stimulating antitumor immunity. Previously we have shown that RA can increase the expression of CD1d on monocytic cells and macrophages. CD1d is related to the a major histocompatibility complex (MHC) class I, but is unique from MHC-I. CD1d is expressed by several types of cells throughout the body, particularly by antigen-presenting cells (APC), such as dendritic cells (DC). CD1d is upregulated transcriptionally by retinoic acid (RA) in THP-1 cells, and murine macrophages (2).



CD1d is known to be a direct factor in the stimulation of natural killer T cells (referred to as NKT cells, invariant (i)NKT cells, CD1d-dependent NKT cells, or V β 8-expressing NKT cells (3-5). The binding of α GalCer to CD1d on antigen-presenting cells (APCs) leads to the activation of iNKT cells, leading in turn to increased cytokine production (interferons, IFNs; IL-4 and others). We believe that PIC could potentially further augment the production of cytokines by DC or other cells, including the release of type I and type II IFNs that enhance innate antitumor immunity. Based on this reasoning we hypothesized that the triple combination of 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 (6). 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 (2). A depiction of our hypothesis is shown in [Fig. 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 in our application. A potential advantage is that the initial treatment of the cancer patient's own immune cells (APCs such as DC or B cells that can be collected as autologous blood cells) could be done outside the body by activation in culture, and then reintroduced after treatment. This ex vivo treatment would have the advantage of minimizing the exposure of the patient to drugs (RA, α GalCer) that, if given systemically, could result in unwanted side effects. 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.

Key research accomplishments in year 2

At the end of year 2 we had conducted up to tasks 10-12 of our SOW. The exact order of experimentation was modified somewhat as results were obtained. As noted last year, we stopped using small animal magnetic resonance imaging (saMRI) for tumor detection as it was not sensitive enough and we found that palpation was equally effective. We also added a test for micrometastases to assess the spread of 4T1 cells to the lungs. We focused on testing the combination of RA and α GalCer-treated DC rather than on testing each treatment separately and both combined, as we believe that once we have evidence for a positive effect of the RA/ α GalCer combination, then we can take the combination apart to understand which of the components is effective or if both are required. Results for year 2 are described in the next section, followed by our plan for year 3 (completion of tasks 13-18 including manuscript preparation).

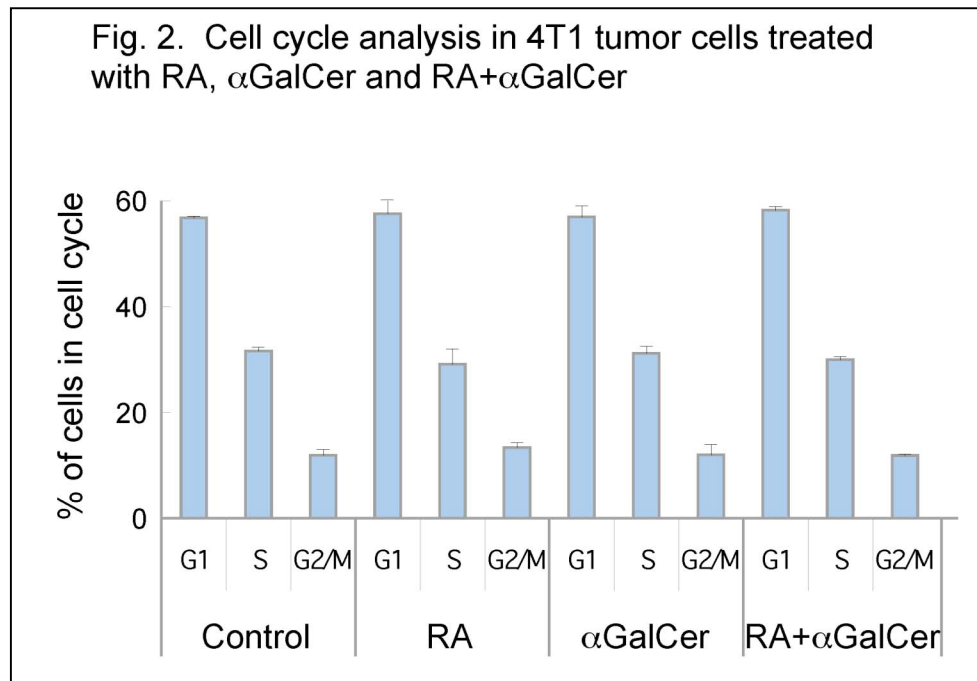
A summary of the main findings is:

- 1) The combination of RA and α GalCer did not act directly on 4T1 cells in vitro. This result thus has focused our attention on how their actions are mediated through other cells.
- 2) In vivo, the 4T1 tumor itself induced splenomegaly and altered splenic lymphocyte populations, particularly a reduction in CD3-CD8 T cells and an increase in granulocytes.
- 3) In vivo, mice that received 4T1 cells and that received DC treated ex vivo with the combination of RA, used to induce CD1d, and α GalCer to bind to CD1d and activate NKT cells (as reported last year), had a smaller increase in spleen size and weight. Treatment with DC also moderated immune activation as compared to mice that received 4T1 cells only.
- 4) The 4T1 mammary tumor size was reduced in the mice that received the ex vivo activated (RA/ α GalCer-treated) DC, compared to mice with 4T1 tumor only.
- 5) A limitation of the results so far is the small number of mice. The studies from year 2 must be repeated before statistical analyses. Additional studies in vivo are indicated.

1. RA and α Galcer did not directly affect 4T1 cell growth

Because in our model we will treat the APC (DC) with RA to induce CD1d and with α GalCer as an activator of iNKT cells, we need to know whether these agents directly affect the growth of 4T1 cells, or whether the effect if any is more likely due to the mediation of treated DCs. Therefore we tested whether RA and α GalCer directly affect 4T1 cell growth in vitro. 4T1 cells were cultured in the presence and absence of RA (20 nM) and α GalCer (100 nM) for 24 h. Two tests were conducted. First, [3 H]-thymidine was added to medium for the last 4 hours (n=6/treatment). DNA synthesis was measured by scintillation counting. Second, in a separate set of cells, 4T1 cells were harvested and fixed with 80% ethanol. Propidium iodide staining was performed (reagents from BD Biosciences) and cellular DNA amount was determined by flow cytometry (FCS Express, De Novo Software, Los Angeles, CA) to measure the distribution of cells in the G1, S and G2/M phases of the cell cycle. Treatment with RA and α Galcer did not change the cell proliferation rate as measured by [3 H]-thymidine incorporation (data not shown). This was confirmed by cell cycle distribution analysis (n=3 wells of cells/treatment group), which also did not show any difference in the percentage of cells in G, S, or G2/M of the cell cycle among the different treatments (Fig. 2). These data suggest that even though RA and α Galcer have both been shown to reduce the growth of some tumor cells, they do have a direct effect on the growth of the 4T1 breast tumor cells. From these results we can conclude that if changes in the growth of the 4T1 tumor cells are observed with RA and α Galcer treatment in vivo, they

will be most likely to be mediated through activation of other cells and not directly on the growth of 4T1 cells.



2. RA and α GalCer treated DC reduced the 4T1 cell growth and metastasis in vivo

26 day study

Dendritic cells were isolated from spleens of adult Balb/c mice by positive selection of CD11c cells (StemCell Technology DC isolation kit). After 4 passage through the isolation column, the DC population comprised 80% of the total cells. This is a good enrichment for this rare cell type. Sufficient DCs were obtained from 3 donor mice to treat the remaining experimental mice with DCs, given by the i.v. route, at the time of the tumor inoculation which was given subcutaneously (s.c.) into the right mammary fat pad. The DCs were first treated ex vivo with RA (20 nM) for 16 hours (overnight) and then treated again with RA, TNF α (5 ng/ml) and α GalCer (100 nM) for 2 hours the next morning. The DCs were then washed two times. An aliquot was saved for characterization by flow cytometry. Then 1×10^5 cells in 100 μ l of PBS were injected i.v. into the recipient Balb/c mice. At same time, 4T1 breast tumor cells (2×10^4 cells/100 μ l in PBS) were injected s.c. into the mammary fat pad. The mice were monitored every day. Mice were euthanized when the tumor size at the injection side grew to ≤ 1 cm (about 26 days after injection/inoculation). Mice without any injection of tumor cells were included as extra controls. Comparisons were made between the group that received

the 4T1 cells s.c. without DC injection, while the treatment group received pulsed DC i.v. at the time the 4T1 cells were injected s.c. In this study, there were n=2 untreated mice, n=2 mice with DC only (no tumor) and since these showed no differences we have combined them as the control (for spleen weight measurement). There were n=6 mice for the 4T1 only group and n=6 for the 4T1 + DC group. One mouse was accidentally lost from the 4T1 group, so that the final n was 5. We realized from the outset that the experiment would need to be repeated with more mice before final statistical results could be calculated. Thus the statistics shown are preliminary and are provided only to signify the potential differences that might be obtained by the treatment.

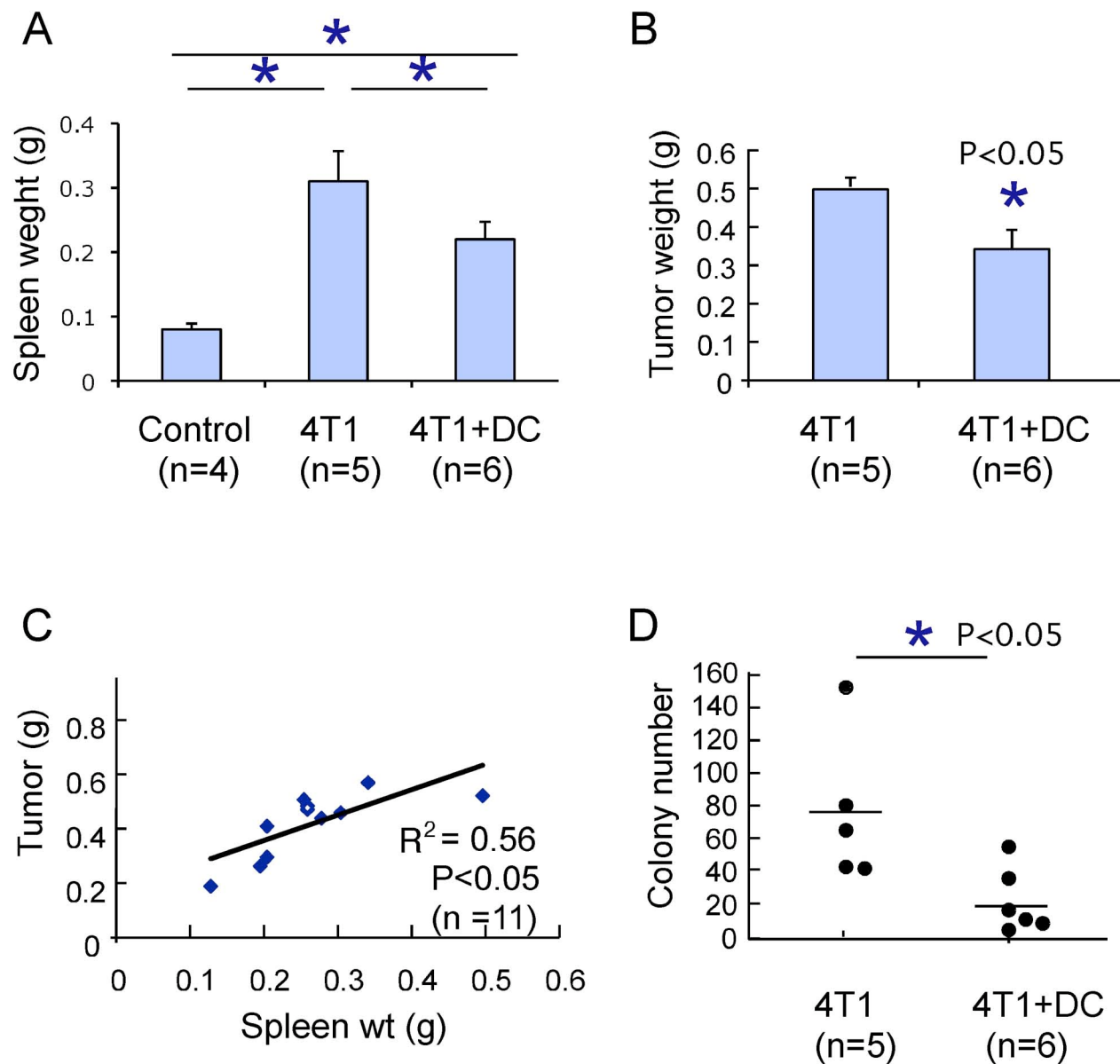
The mice that received the 4T1 cells started to show mammary tumors at the injection site about two weeks after injection. At first, these were “sandy feeling” tumors, palpable only as a slight toughness under the skin. At the time of dissection, day 26, tumors in nearly all of the mice were well localized as tight nodule(s) without local invasion. The tumors and other organs such as spleen, liver and lung were carefully dissected and weighed. Body weight as well as liver and lung weight did not differ among the treatment groups; however, a marked difference was noted regarding spleen size and weight. As shown in Figure 3A, 4T1 cell injection caused a significant enlargement of the spleen. Splenomegaly in the 4T1 model was reported previously (7). It is interesting to note that with the injection of RA-treated, α GalCer pulsed DCs, the spleen weight was reduced although not back to the normal level. The same pattern was observed for tumor weight, as DC injection decreased the weight of the local tumor (Fig. 3B), implying that growth was slowed. The correlation between spleen and tumor weight was significant ($R^2 = 0.56$, $P < 0.05$), shown in Fig. 3C.

We then digested the lung and cultured the single cell suspension in the medium with 60 μ M of 6-thioguanine, which suppresses the growth of lung cells but not of the 4T1 cells. After 14 days of culture, the 4T1 cell colonies were fixed with methanol and stained with 0.03% methylene blue dye for counting. The number colonies is presented in Fig. 3D. Injection of pulsed DC resulted in a considerable reduction in the 4T1 cell colony number, suggesting a decreased metastasis rate.

Figure 3. 26 day study.

Spleen weight, mammary gland tumor weight and number of meta-
 static cells in lung of mice treated with RA/ α GalCer-pulsed DC and
 inoculated with 4T1 breast cancer cells.

(* = $P < 0.05$ in preliminary statistical testing)



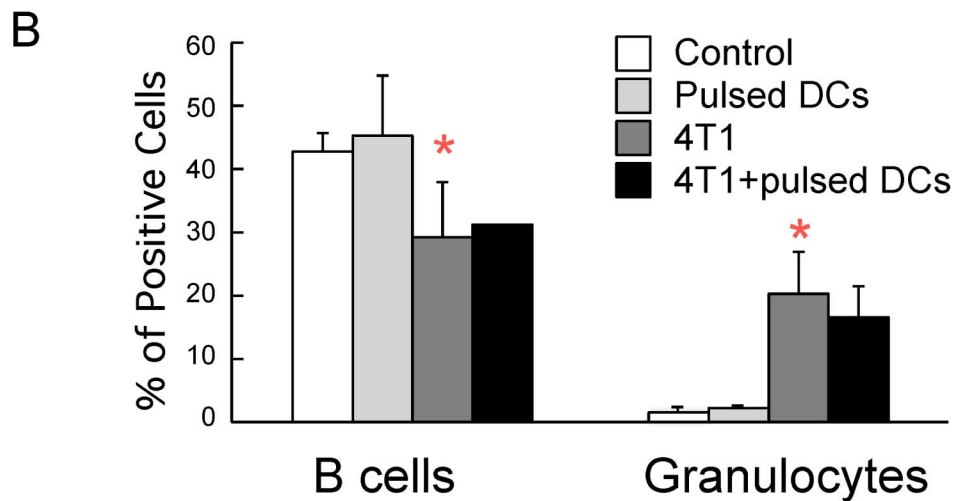
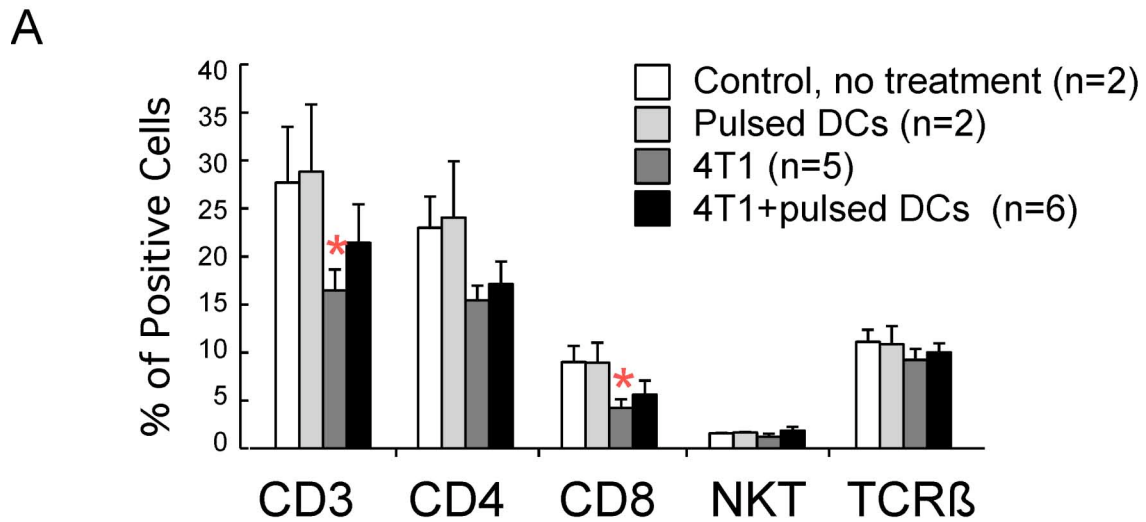
In this 26-day experiment, we also processed the spleen and tested for major cell populations as an indicator of immune activation. As shown in [Fig. 4A](#), the total T cell (CD3 positive) and B cell (CD19 positive) populations in the group of mice that received 4T1 cells only were both dramatically lower compared to the control groups without 4T1 cells, while the addition of the RA/ α GalCer-pulsed DCs tended to lessen the reduction in immune cells (no longer significantly lower in the 4T1+DC group). (For the statistical analysis we combined the Control and DC only groups because neither received tumor and the results were very similar. We compared this group to the 4T1 tumor group, and the 4T1+DC group.) The reduction in CD3 T cells was located in the CD8 T-cell compartment, as it showed a similar reduction to CD3 T cells in the 4T1 group and a partial reversal in the 4T1+DC group. No change was observed in the NK cell or NKT (V β TCR) cell populations. B cells were reduced by the tumor but DCs did not correct this change ([Fig. 4B](#)). However in contrast to the reduction in T cells by 4T1 cells, the granulocyte population was markedly increased in the 4T1 tumor group ([Fig. 4C](#)), which was again partly brought down by treatment with DCs, although this was only a trend.

These data suggest that the 4T1 tumor cells caused changes in the spleen cell populations, with the reduction of T and B cells and increase of granulocytes. Co-injection of RA/ α GalCer pulsed-DCs could counteract some of the effects that the 4T1 cells had on splenic lymphocyte populations, which was apparently significant for the CD3 and CD8 (cytotoxic) T cell populations.

Since T cells and especially cytotoxic T cells are likely to be important in immune surveillance against tumors, we consider these results encouraging. A limitation is that the n/group is still small and thus the study needs to be repeated.

Figure 4. 26 day study.
 4T1 tumor cells suppress, and RA/ α GalCer-treated (pulsed) DCs partially restore the number of CD3 and CD8-positive cells in spleen.

Mean $\pm$ SE; * indicates $P < 0.05$ vs. combined controls

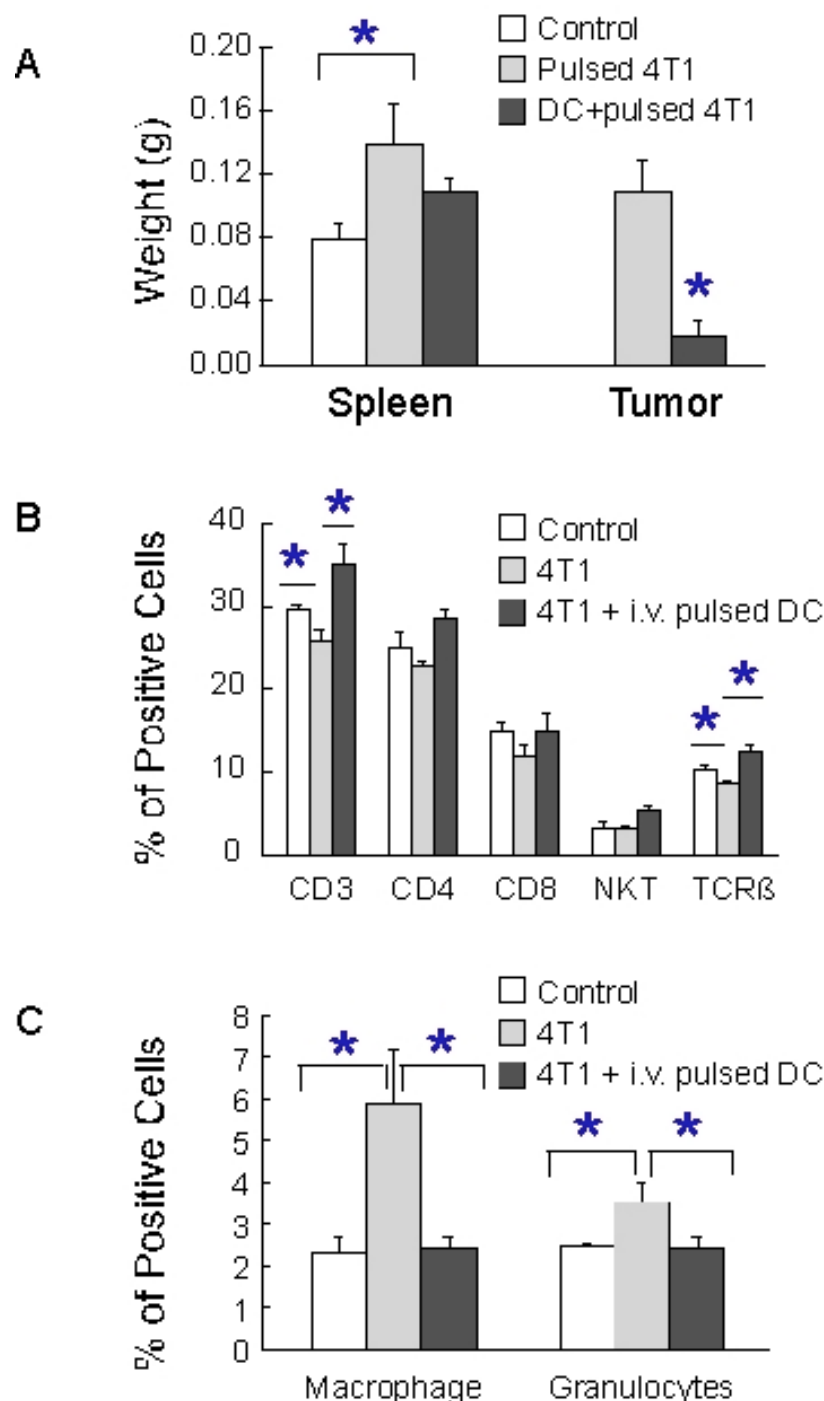


18-day study

Because the splenomegaly produced by the 4T1 cells in the 26-day experiment was quite overwhelming (>3 fold weight increase) and the effect of the DCs was partial in normalizing spleen weight and the spleen cell populations, we performed a similar experiment for a shorter time in which tumors and spleen were collected 18 days after inoculation with 4T1 cells and i.v. injection of DC. We thought that with the shorter time the tumor might not be so overwhelming and might be more sensitive to the pulsed DC. The size of the local tumor at the time of dissection was palpable but much smaller than in the 26-day experiment above (about 2-3 mm in diameter or less). The splenic enlargement caused by the 4T1 cells was still significant ([Fig. 5A](#)), although the weight was only about half that in the previous experiment of 26 days (compared with [Fig. 3A](#)). Nevertheless we observed again that injection of pulsed DC tended to decrease the 4T1 cell-caused splenomegaly. Even though the mammary tumors were generally small in the 4T1 group, i.v. injection of DCs significantly decreased the local tumor growth in the mammary gland. Changes in spleen cells were also observed in a pattern that was similar to that observed in the previous experiment, with pulsed DCs reducing the increase in spleen size due to the 4T1 tumor. 4T1 cells again decreased the T cell population of the spleen, including NKT cells ([Fig. 5B](#)), and markedly increased the macrophage and granulocyte components ([Fig. 5C](#)). However, the percentage increase due to the 4T1 tumor in this 18-day experiment was much less than the previous 26-day experiment (compare [Fig. 5B](#) and [4B](#)). Yet the same pattern of reduced CD3 T cells was found, with a normal percentage in the 4T1 group that received the pulsed DC group. A change in the proportion of B cells was not observed (about 45 to 50% in all of the groups, which is a normal percentage). Since B cells are the major population in the spleen, the increase in the granulocyte and macrophage population in the shorter time span of this experiment probably was not enough to alter/suppress the B cell population significantly.

Regarding the metastasis assay, although 4T1 cell colonies were detected in lungs of the 4T1-treated group, the colonies were much fewer than the previous experiment. The DC-injected group had no detectable colonies. Thus, while this shorter experiment indicated a similar pattern to the 26-day study, we now believe that we should use the longer experimental protocol, including multiple DC treatments, to further test the effects on DC of RA and α GalCer treatments in this mouse model.

Fig. 5. 18 day study. Spleen weight, tumor weight, splenic lymphocyte populations including macrophages and granulocytes, in mice inoculated in mammary fat pads with 4T1 cells and treated i.v. with pulsed DC



Overall, these experiments suggested that 4T1 breast tumor cell injection could cause a systemic change in the immune system at an early stage, such as 18 days as shown above. The experiments demonstrated the effectiveness of pulsed-DC on the local tumor growth and lung metastasis. Nonetheless, the experiments need to be repeated to increase the sample size before we can prepare a publication, and therefore our plans for year 3 include repeating the 26-day study. The results this year also indicated that the development of a systemic immune response and the extent of metastasis depend on the local tumor growth, as tumor size and splenic response were correlated. To detect a full change of the spleen cell population and to test the effect of DCs on the 4T1 tumor metastasis, a longer time (more than 18 days) is required.

Plan for year 3

In vitro studies

1. Our data so far have shown that there is no direct effect of RA and α GalCer on 4T1 cell growth. However, since 4T1 cells express several cytokine/chemokines, such as G-CSF/GM-CSF, CCL2 and CCL5 (8), that play important roles in the tumor-induced systemic response, immune suppression and tumor metastasis, we plan to detect the cytokine expression level in 4T1 cells treated with RA and /or α GalCer by qRT-PCR and ELISA. If we find that RA and α GalCer do not affect these, then we will have three lines of evidence (3 H-thymidine incorporation, cell cycle analysis, and cytokine expression) all indicating that our treatment agents are not acting directly on the tumor cells.
2. We also plan to test whether DCs that have been treated with RA, pulsed with α GalCer and washed can directly affect 4T1 cell growth by using co-culture or trans-well culture systems. As both DC and splenic B cells each can present α GalCer, which will activate NKT cells, we will also test the effect of pulsed splenocytes on 4T1 cell growth.

In vivo studies

1. We have demonstrated in a preliminary manner that injection of RA-treated and α GalCer pulsed DCs has a protective effect on tumor growth and metastasis, as well as a systematic alteration such as on 4T1 cell-induced changes in spleen cell population. We would like to study the effect of DCs treated by RA or α GalCer alone by using the same animal model. The time span will be about 26 days---by monitoring the mammary tumor size between 5 - 10 mm diameter.
2. We also plan to test whether additional oral dosing of RA and injection of α GalCer in vivo, or additional treatment with pulsed DC will be more effective in addition to the primary treatment with DCs. In this experiment, we will give oral dose of RA for 6 constitutive days, and two doses of α GalCer (100ng/animal, i.m.) injections with an

interval of three days, or a second dose of pulsed DC on day 10. The experiment will end once the mammary tumor reaches 5-10 mm in size.

3. We will also introduce Poly-I:C in this experiment to test whether stimulation of IFN production reduces tumor growth. This design is represented in task 15 in our proposal:

In vitro = factor 1:	Transferred DCs without treatment;	Transferred spleen DCs from best treatment in aim 2 (e.g., RA+ α GalCer)
In vivo = factor 2:	Control (oil p.o., saline i.p.)	Control
	RA (50 μ g in 10 μ l oil /d p.o., days 1-5)	RA (50 μ g/d p.o., days 1-5)
	α GalCer, 2 μ g i.p. day 2	α GalCer, 2 μ g i.p. day 2
	Poly-I:C, 2 μ g i.p. day 2	Poly-I:C, 2 μ g i.p. day 2
	All three: RA+ α GalCer+Poly-I:C	All three: RA+ α GalCer+Poly-I:C

Procedures:

Inject 4T1 breast cancer cells.

One day later, collect spleens and prepare single cell suspension from 5 donor mice as described by (1). Divide into two aliquots for in vitro treatment: 1) vehicle only; 2) the treatment that reduced tumor growth most in aim 2.

Inject treated splenic DCs i.v. as per aim 2.

Task 16 (Month 25): Deliver in vivo treatment (factor 2 in table).

Task 17 (Months 25-26): Monitor the first 30 mice in aim 3, as described in aim 2.

Task 18 (Months 27-29): Process for histology. Collect tumor and adjacent breast tissue; freeze for IRF-1 protein analysis (9).

Reportable outcomes

We have not yet reported our results because we need to repeat the main experiments. We presented data in abstract form at the Era of Hope meeting in Baltimore in 2008. A copy of our Era of Hope abstract is as 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
Nutritional Sciences, Pennsylvania State University, University Park PA, 16802

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 -galactosylceramide (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 γ 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 γ .

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 γ 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.)

Conclusions

At the end of year 2, we conclude that the basic idea proposed in this award has merit, based on an observed reduction in tumor weight and correction of some of the immune dysfunction caused by the 4T1 tumor, when RA-treated α GalCer-pulsed DC were administered to Balb/C mice inoculated with 4T1 breast tumor cells. Although the results are encouraging and we are cautiously optimistic, the experiments need to be repeated for confirmation and complete statistical analysis. If the combination of RA and γ GalCer improves DC function, then we will test RA and γ GalCer separately to determine which component is effective or if both must be given to enhance DC function. Our goals and plan of work for year 3 address these questions.

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