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14. ABSTRACT Our goal was to determine the effects on metastasis of deleting or inhibiting the receptor for interleukin-4 from breast tumor cells. We proposed three aims: [1] Establish and characterize mammary tumor lines with the receptor for IL4 knocked down. [2] Determine the in vivo growth characteristics of IL4R-knockdown cells using spontaneous and experimental metastasis models; [3] Determine the therapeutic efficacy of IL4 neutralizing antibody in combination with chemotherapy in a spontaneously-metastasizing model. We have completed all aims with the mammary tumor cell line 4T1, and obtained confirmatory data for aims 1 and 2 in a second cell line, PyVT-R221a. In vitro assays have indicated that IL4 receptor can promote proliferation and survival. When injected orthotopically in vivo, 4T1 mammary tumors showed no difference, whereas with PYVT-R221A cells, knockdown of IL4R completely abrogated tumor take. For both cell lines, metastatic tumor volume was significantly attenuated when tumor cell IL4R was knocked down. The difference in behavior of primary mammary tumors may reflect the more aggressive properties of the 4T1 line. Disappointingly, combination therapy using paclitaxel with an IL4 neutralizing antibody showed no benefit in the 4T1 metastasis model. This was most likely due to ineffective dosing of agents, especially paclitaxel.					
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Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	10
Reportable Outcomes.....	10
Conclusions.....	11
References.....	n/a
Appendices.....	12

INTRODUCTION

Cancer cells that persist after chemotherapy are especially dangerous to patients as they can seed new tumor outgrowths. Interleukin-4 (IL4), a cytokine known primarily for its immune system functions, has been proposed as a survival factor for breast cancer cells, and thus disabling its function provides a possible method to ablate these insidious tumor cells. The hypothesis we wished to test therefore was :**Blocking IL4 signaling will attenuate breast tumor cell survival and, in combination with chemotherapy, will promote disease eradication.** We proposed three aims to test this hypothesis: [1] Establish and characterize mammary tumor lines with the receptor for IL4 knocked down. [2] Determine the *in vivo* growth characteristics of IL4R-knockdown cells using spontaneous and experimental metastasis models; [3] Determine the therapeutic efficacy of systemic IL4 neutralizing antibody in combination with chemotherapy in a spontaneously-metastasizing breast cancer model.

BODY

The goal of the project is to determine the effects on metastasis of deleting or inhibiting the receptor for interleukin-4 from breast tumor cells. The primary method chosen for deleting expression of the gene was shRNA-mediated knockdown. We proposed to use 2 different murine mammary tumor cell lines for this work, as it is important that studies not be an artifactual finding in a single cell line. Furthermore, within each cell line, we proposed using several clones with different shRNA targeting sequences to minimize the possibility of artifact or off-target effects. In the 4T1 cell line, we have successfully generated 2 knockdown clones for each of 2 different targeting sequences, and one clone with a third targeting sequence. After several attempts and different targeting systems, we have now also obtained several knockdown lines in the PyVT-R221A cell line.

Following, we detail the progress for each task originally defined in our statement of work.

Task 1: Obtain regulatory approval for animal experiments

All regulatory approvals were received before the commencement of the project.

Task 2: Generate and characterize mammary tumor cell lines without IL4R

2a: Use commercially available lentiviral shRNA particles to infect cultures of 4T1 and PyVT-R221A cells and select clones with puromycin.

2b: Analyze levels of knockdown in various clones by RT-PCR and western blotting

This task, which was scheduled to take 2 months, actually took 4 months to complete for the 4T1 cell line; and 18 months for the PyVT-R221A cells.

Lentiviral shRNA particles generated against 4 different sequences of murine IL4R α (Sigma, St Louis, Mo) were used to infect cultures of both 4T1 and PyVT-R221A cells. After selection with puromycin, multiple colonies were obtained for both cell lines and with 3 of the 4 shRNA sequences.

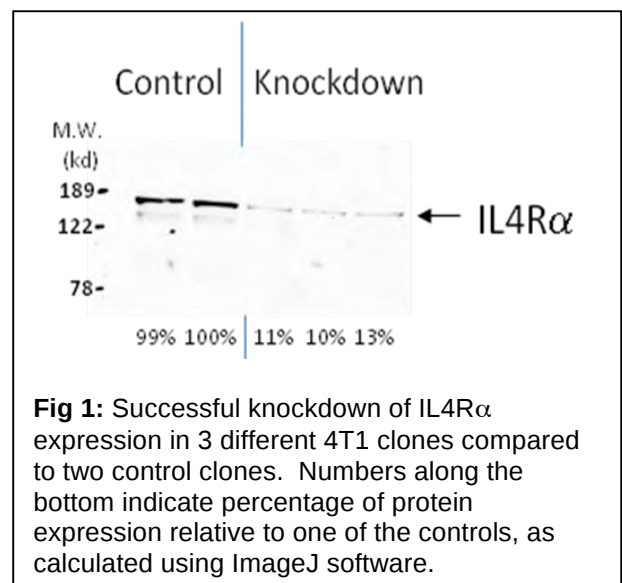
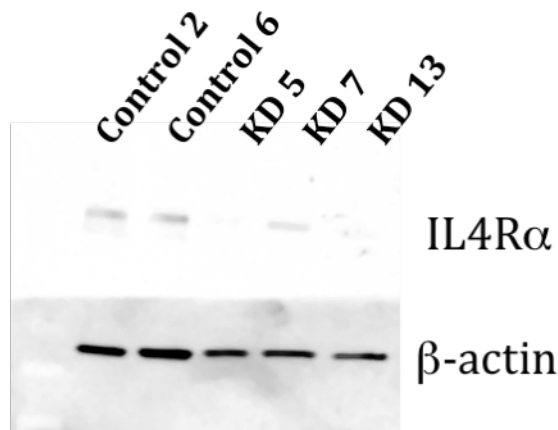


Fig 1: Successful knockdown of IL4R α expression in 3 different 4T1 clones compared to two control clones. Numbers along the bottom indicate percentage of protein expression relative to one of the controls, as calculated using ImageJ software.

One of the sequences never resulted in puromycin-resistant colonies. Colonies were picked and expanded in puromycin-containing medium and then stocks were frozen. Of 30 4T1 clones examined (10 for each of the three successful targeting sequences), 5 showed a protein knockdown of greater than 50%. The top three, each representing a different targeting sequence were selected for further use. These clones: 188.2; 189.8 and 190.4 showed knockdown of 75-90% at the protein level, which was confirmed at the transcript level. Two control clones which had been infected with a scrambled non-targeting shRNA were also selected for further use. As expected, these control clones (C3 and C5) showed no change in IL4R α level. Figure 1 shows both the control and knockdown clones as analyzed at the protein level using a specific antibody for IL4R α .

Multiple colonies(>25) of PyVT-R221A cells exposed to the same lentiviral shRNA particles were expanded and similarly tested by both western blotting and RT-PCR. Despite showing puromycin resistance, none of the clones showed more than 30% knockdown when analyzed, which was not regarded as useful. A different lentiviral system was purchased and infections of PyVT-R221A cells repeated as above with the new particles. Unfortunately, the system contained a polyclonal mixture of shRNA targeting sequences so we were not able to specifically isolate sequence-specific knockdown clones. However, the system was successful and we have generated several control and knockdown clones. Figure 2 shows the protein levels of IL4R α in a group of clones indicating successful knockdown. Real-time PCR experiments confirmed a reduction of *il4ra* transcript levels to 35-40% of controls.

Fig 2: Successful knockdown in PyVT-R221A clones (3 lanes on right) versus control clones (2 lanes on left). The band on the lower portion is β -actin to allow normalization of protein levels.

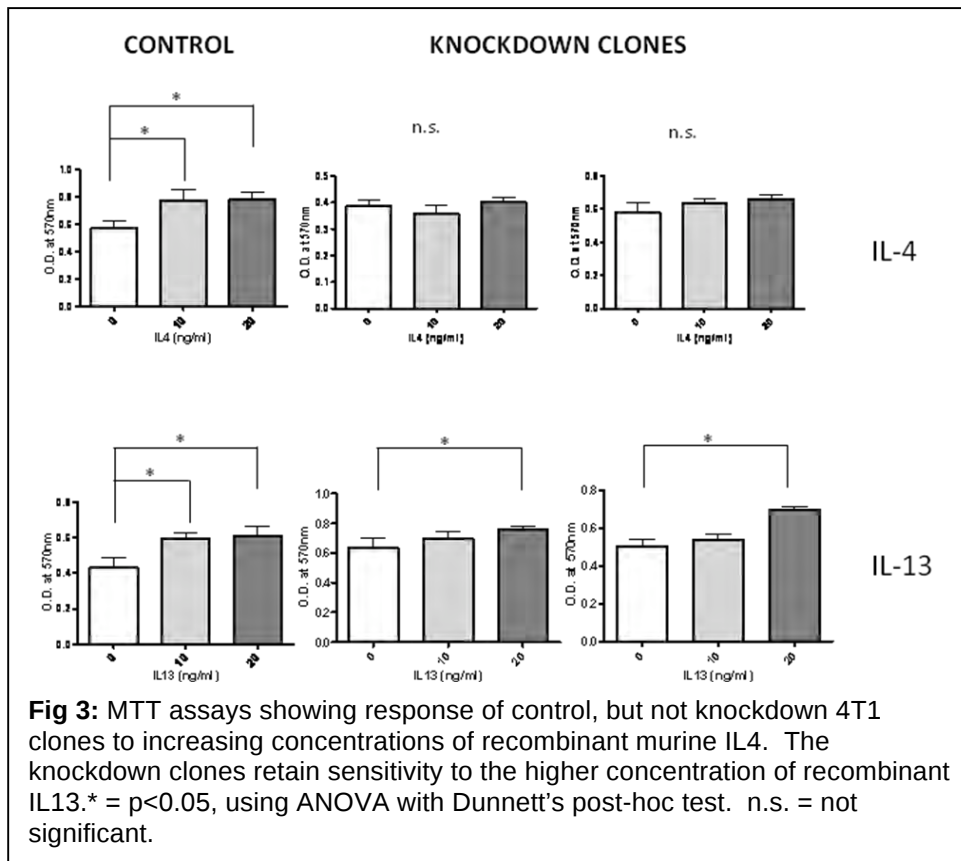


2c: Analyze proliferation and apoptosis rates in vector control vs IL4R α knockdown (KD) clones, both basal and post-paclitaxel exposure.

Baseline proliferation was not different amongst the various 4T1 control and IL4R α -KD clones, as assessed by MTT assay. However, control clones responded to increasing concentrations of exogenous recombinant IL4 with increased proliferation, but this response was not observed in the 4T1 IL4R-KD clones (Fig 3). This result was expected since the proliferative activity of IL4 requires signaling through IL4R α . Interestingly, the IL4R α -KD clones retained sensitivity to increasing

concentrations of IL13 (Fig 3). IL13 is a related cytokine to IL4 and most of its signaling is also thought to require IL4R α . There is a second version of an IL13 receptor that does not contain IL4R α , however whether is a functional receptor or merely acts as a decoy is not clear from the literature. Our results suggest the second IL13 receptor may be functional, at least in 4T1 cells.

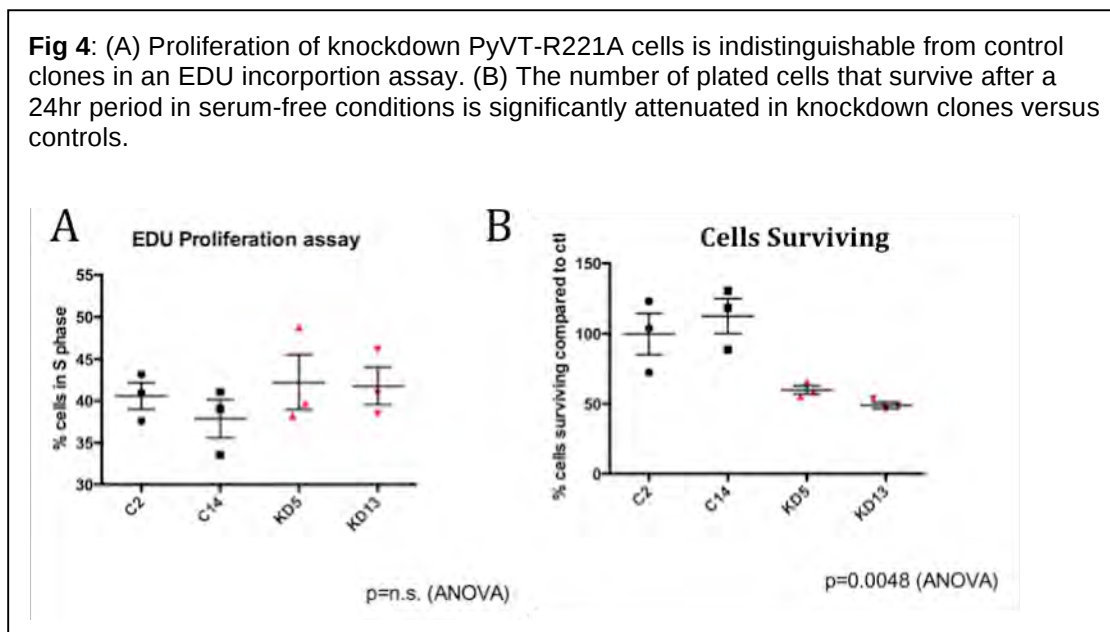
To assess apoptosis and proliferation post-paclitaxel, we initially used MTT assays and varying doses of paclitaxel. 4T1 cells are sensitive, but not at low doses. Using MTT assays, a dose of 5 μ M paclitaxel was identified as the IC₅₀ for the control clones, however 2 of 3 of the IL4R α -KD clones required 1-2 μ M to kill 50% of the cells. This is in the absence of exogenous IL4 or IL13.



In the PyVT-R221A cells, an EDU assay (similar to BrDU incorporation for measurement of cells in S phase of cell cycle) clearly established that there was no effect of IL4R α knockdown on DNA synthesis, an

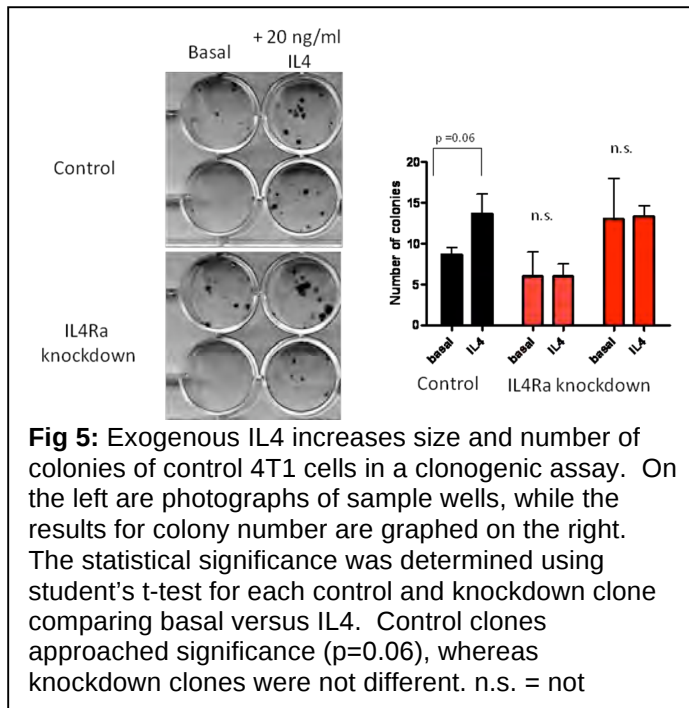
indicator of proliferation rate (Fig 4a). However, there was a significant change in the number of cells alive after a 24hr period (Fig 4b). These data suggest that in this cell line, the dominant role of IL4R α is in survival signaling.

2d: Analyze clonogenicity using soft agar assays of vector control versus KD clones, both basal and post-paclitaxel exposure.



Under basal conditions, clonogenic survival was similar amongst control and IL4R α -KD clones. When recombinant IL4 was added, there was an increase in colony size (indicating a proliferative

effect) and colony number (indicating a survival effect) in the control clones (Fig 5). IL4R α -KD clones showed no change in size, although there was a small increase in colony number in some wells leading to high variability (Fig 5). This may suggest that survival is very sensitive to signaling through IL4R α , and even with only 10-25% IL4R α protein remaining, this is sufficient to confer increased survival ability in response to IL4.



The addition of paclitaxel to the clonogenic survival assays was not as straightforward as anticipated, as the doses identified by MTT assays as IC50s allowed for no surviving colonies. We thus re-did dose-response curves to paclitaxel using clonogenic survival as the readout. In the absence of IL4, 0.5 μ M was identified as the dose irrespective of IL4R α -KD status. In the presence of IL4, control clones exposed to paclitaxel demonstrated more colonies than did IL4R α -KD clones under the same conditions. However, after statistical analysis the differences were not found to be significant for any clone due to a high degree of variability. Our data suggests that signaling through IL4R α does

appear to confer a survival advantage, both at baseline and after exposure to paclitaxel, but repetition will be required to fully demonstrate this effect. If our hypothesis holds true, we believe that inhibitors of the IL4R α pathway may be clinically useful to prevent survival of tumor cells during chemotherapy.

Task 3: Determine *in vivo* growth capability of control vs KD clones

3a: Perform orthotopic implantation of 4T1 control and KD cells into 6-week old female BALB/c mice (8 per line). Sacrifice after 5 weeks and harvest tissues.

We mixed the control clones together to make a polyclonal control, and similarly the KD clones together to make a polyclonal KD cell inoculum. The polyclonal cell populations were used for all *in vivo* experiments.

With the 4T1 cells, no difference was seen control and KD cells in the mammary gland tumors (Fig 6a).

3b: Perform tail vein injection of 4T1 control and KD cells into 6-week female BALB/c mice (8 per line).

As above, polyclonal mixes of either control or KD cells were used and injected into the lateral tail veins of recipient mice. The lungs of mice injected with KD cells had fewer and smaller tumors than those injected with control cells (Fig 6b).

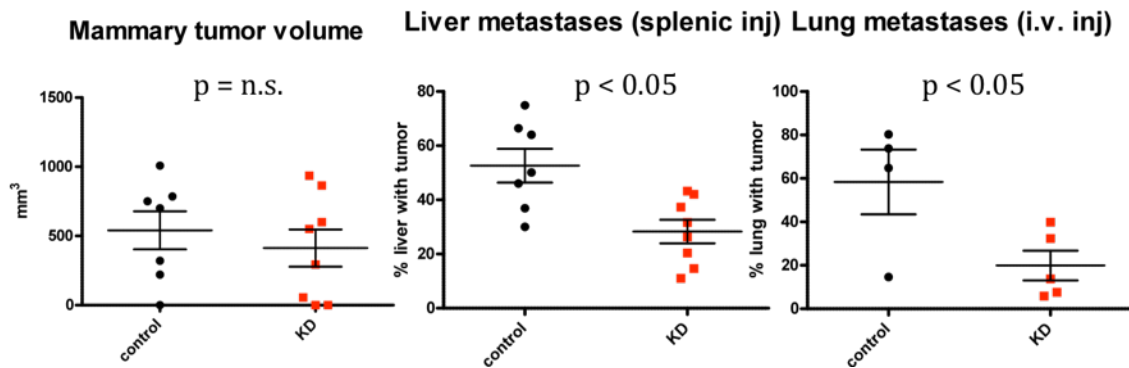
3c: Perform intrasplenic injection of 4T1 control and KD cells into 6-week female BALB/c mice (8 per line).

Again, polyclonal mixtures were used for injection. As seen with the lungs, livers of mice that received KD cells had lower tumor burden than those that received control cells (Fig 6c).

3d: Perform intra-tibial injections of 4T1 control and KD cells into 6-week old female BALB/c mice.

Due to some technical difficulties with the instrumentation required, analysis of the bones is still ongoing. Preliminary results suggest a similar reduction in tumor burden with the KD cells, however the numbers analyzed are too low as yet to determine if the results are significant.

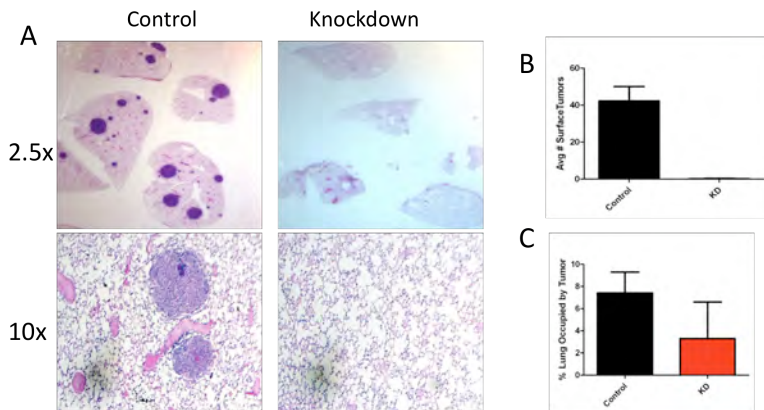
Fig 6: Tumor burden after in vivo injection of 4T1 control and knockdown polyclonal mixtures in (A) mammary glands; (B) liver via the spleen; and (C) lungs via tail vein.



3e-g : Injections of PyVT-R221A control and KD clones.

Similar experiments to those described for the 4T1 cells were also performed with the PyVT-R221A control and *il4ra* knockdown clones. In this case, the orthotopic mammary gland injections showed no tumor take with any of the KD injections, whereas all controls had tumors. Tail vein and intrasplenic injections also showed significant attenuation of growth at metastatic sites (e.g. Fig 7). The results of the intratibial injections await analysis.

Fig 7: (A) Examples of lungs from mice injected with control (left) or knockdown (right) PyVT-R221A cells. (B) Quantitation of surface tumors on lungs injected via the tail vein with control or knockdown PyVT-R221A cells. (C) Quantitation of lung section area occupied by tumors.



3h-j: Analysis of tissue

The immunostaining for cleaved caspase-3, a marker of apoptosis, and for Ki67, a marker of proliferation, has been completed. The quantitation of the amount of apoptosis and proliferation is still ongoing.

Task 4: Determine efficacy of systemic IL4 neutralization +/- chemotherapy in 4T1 model.

Fig 8: Experimental groups for testing anti-tumor effects of systemic IL4 neutralization with or without the chemotherapeutic agent paclitaxel. All mice were injected at 6 weeks of age with 1×10^6 4T1 cells into the #4 mammary gland. Once tumors were palpable, the indicated treatments were initiated. Groups 1-4 were terminated once the 4-week treatment period ended. Groups 5 and 6, although planned to continue for 2 further weeks as shown, were terminated one week after treatment ended due to disease extent.

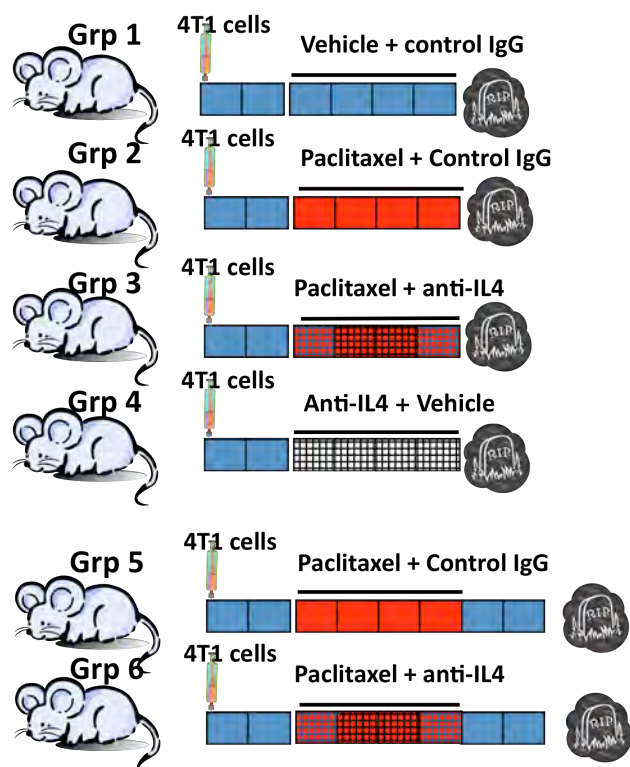
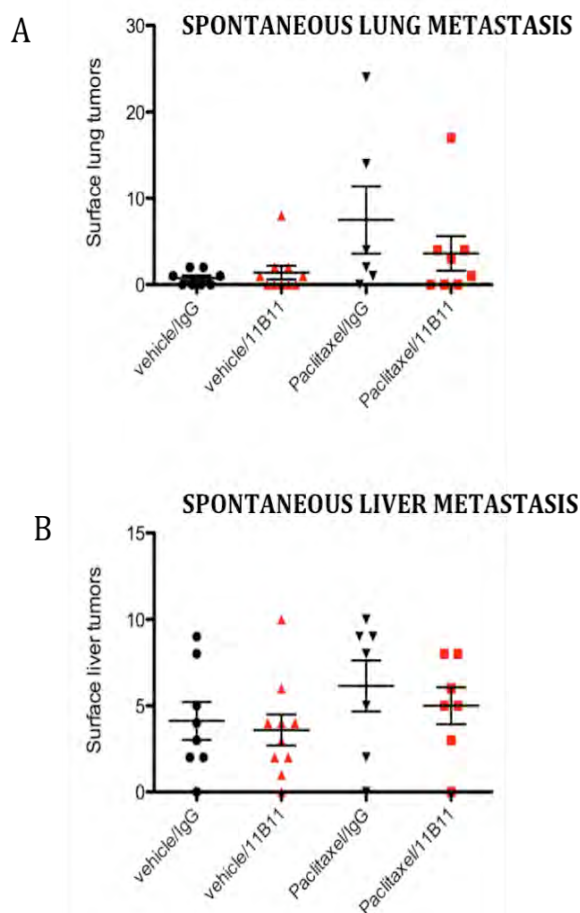


Fig 9: Tumor burden in (A) lungs and (B) livers of animals from groups 1-4 as shown in Fig 8.



Since knockdown of the receptor for IL4 in the breast cancer lines 4T1 and PyVT-R221a resulted in reduced tumor burden in both spontaneous and experimental metastasis assays as described above, we performed a more clinically relevant test of IL4 blockade with a neutralizing antibody in combination with paclitaxel. This was done with the 4T1 spontaneous metastasis model, and dosing of both paclitaxel and 11B11, the IL4 neutralizing antibody, (or appropriate controls) was according to published literature. Figure 8 shows the treatment groups. For this experiment, we divided up the total mouse number (proposed to be $n=14$ per group) into 2 ($n=10$ and $n=4$) to ensure all injections and measurements could be easily completed by a single researcher in a reasonable time. All mice were injected orthotopically with 4T1 cells and not treated until tumors were palpable in all (after 10 days). To our surprise, after 4 weeks of treatment, there was no apparent effect of either paclitaxel or 11B11 either alone or in combination. In all treatment groups, it was necessary to remove one or more mice during the treatment period as large

tumors necessitated euthanasia. Fig 9 shows the numbers of metastatic lesions in lungs and livers of mice from each of the 4 treatment groups terminated immediately post-treatment. Quantitation of spontaneous metastasis to bone is still being completed. The initial plan called for two groups of mice to be monitored for 2 weeks after treatment ended, however because of large primary tumor sizes in these mice despite all receiving paclitaxel treatment, these animals were terminated after only 1 additional week. Complete quantitation of the lesions in these mice is still underway.

Given the disappointing results, we decided not to inject the remaining 4 mice per group, and will instead design a new experiment using abraxane treatment with or without IL4 neutralizing antibody. This will be performed using institutional funds.

Task 5: Write and submit manuscript describing these studies.

This task is currently ongoing with a predicted completion date of Dec 31st, 2011.

KEY RESEARCH ACCOMPLISHMENTS

[1] Have successfully obtained multiple IL4Ra-knockdown clones of the murine mammary tumor cell lines, 4T1 and PyVT-R221A.

[2] *In vitro* assays with 4T1 cells indicated that IL4Ra is required for the proliferative effect of IL4, but not of a closely related cytokine IL13.

[3] In PyVT-R221A cells, IL4Ra knockdown was especially associated with loss of survival rather than effects on proliferation.

[4] In vivo assays comparing control or IL4Ra KD 4T1 cells showed no effect on mammary tumors, but significant reductions in tumor growth at metastatic sites.

[5] In vivo assays comparing control or IL4Ra KD PyVT-R221a cells showed significant effects on tumor take in mammary glands and on tumor burden at metastatic sites

REPORTABLE OUTCOMES

Reagents:

Generation of stable clones of IL4Ra-knockdown 4T1 and PyVT-R221A cells

Poster Presentations:

[1] Hwang DG, Dozier EA, Carter KJ and Fingleton B. *Epithelial Interleukin4 receptor promotes metastasis of mammary tumor cells*. American Association for Cancer Research Annual Meeting, Orlando, FL, April 2011.

[2] Venmar KT, Hwang DG, Dozier EA, Carter KJ, Gillani S and Fingleton B. *Inhibition of Interleukin 4, a survival factor for breast cancer cells, as an anti-metastatic approach*. DOD Era of Hope Meeting, Orlando, FL, August 2011.

[3] Gillani S, Venmar KT, Dozier EA and Fingleton B. *Interleukin-4 receptor affects tumor cell proliferation and survival in vitro and in vivo*. Vanderbilt Summer Science Academy Research Symposium, Nashville, TN, August 2011.

Abstracts for 1 and 2 are provided in the appendix.

Training/Research opportunities

[1] **Daniel G. Hwang**, a Vanderbilt undergraduate student, worked on this project as part of a lab placement for academic credit program. Daniel has since gone on to Medical School at University of Texas, Southwestern.

[2] **Sareena Gillani**, an undergraduate student at Emory University, participated in Vanderbilt University's Minority Student Program at the Summer Sciences Academy June-Aug 2011. This program gives minority students access to research experience by matching them with a lab mentor who has them work on a research project. The students also attend seminars and classes on diverse topics such as preparing for GREs, responsible conduct of research, etc. Stipends for the students participating in the program are paid by Vanderbilt Summer Program. Ms Gillani worked on this CDMRP-funded project and presented a poster outlining her work at the research symposium held at the completion of the program. Her poster earned her an award of merit.

[3] As a rotation project in the first year of graduate school, **Katherine T. Venmar**, contributed to these studies. She subsequently joined the Fingleton lab and will continue working on IL4 receptor signaling in cancer as her dissertation focus.

CONCLUSION

Our results support the hypothesis that the IL4 receptor promotes survival of breast cancer cells at metastatic sites. Although not formally demonstrated here, we assume that this is because of active signaling following binding of either the IL4 or IL13 ligands. Future studies will assess the role of each of these ligands individually and determine whether targeting of either ligand or of the receptor would be the best therapeutic approach. We intend to pursue this pathway as a therapeutic target in metastatic cancer, and will partner with other labs, biotech firms and/or the pharmaceutical industry as necessary to develop drugs for pre-clinical and clinical testing.

APPENDIX 1: PERSONNEL RECEIVING PAY DURING PROJECT PERIOD

Barbara Fingleton Ph.D.	Principal Investigator
Kathy J. Carter B.S.	Lab Manager
Ashley Dozier B.S.	Research Assistant
Katherine Venmar B.S.	Graduate Student [for 1 month]

Please note Daniel Hwang and Sareena Gillani also participated in this work, however they received no pay from the grant as they were compensated by student stipends.

APPENDIX 2: MEETING ABSTRACTS

[1] American Association for Cancer Research Annual Meeting, Orlando FL, April 2011

Epithelial interleukin4 receptor promotes metastasis of mammary tumor cells

Interleukin-4 (IL4) is known primarily for its immune system functions where it can stimulate lymphocytes and promote macrophage activation. Various cancer cells, including breast, are now known to express functionally active IL4 receptors, which appear to play a role in cell survival. We believe that blocking IL4 signaling specifically in epithelial cells will attenuate metastasis because of reduced cell survival in secondary sites. To test this hypothesis, we have generated multiple clones of two murine mammary tumor cell line that show efficient knockdown of an essential component of the IL4 receptor, IL4-receptor [IL4R] alpha. Initial characterization of knockdown and control clones showed a significantly slower growth rate in vitro as assessed both by metabolic (MTT) assays and direct cell counting. Flow cytometric analysis using either annexin V staining or sub G1 measurement showed that clones lacking IL4Ralpha had 2-fold higher basal levels of apoptosis. To test effects in vivo, we have performed experimental and spontaneous metastasis assays using clones of the 4T1 cell line. Both pulmonary and hepatic metastatic lesions were significantly decreased [2.4-fold lower] in mice injected with IL4Ralpha knockdown clones compared to controls. There was however no significant effect on the primary mammary tumors. Together these results suggest that IL4 signaling in epithelial tumor cells is pro-metastatic, and blocking it may be a useful therapeutic approach for preventing metastatic spread.

[2] Dept of Defense Era of Hope Meeting, Orlando FL, August 2011

Inhibition of Interleukin-4, a survival factor for breast cancer cells, as an anti-metastatic approach

Background/Objective:

Cancer cells that persist after chemotherapy are especially dangerous to patients as they can seed new tumor outgrowths. Interleukin-4 (IL4) is a cytokine known primarily for its immune system functions, and it is especially important in allergy and asthma. Indeed, drugs that interfere with IL4 function are in development for these conditions. Various cancer cells, including breast, are now known to express functionally active IL4 receptors. IL4 signaling through these receptors has been proposed as a survival factor for cancer cells. Our hypothesis is that: **Blocking IL4 signaling will attenuate breast tumor cell survival and, in combination with chemotherapy, will promote disease eradication.** In particular, we believe that blocking IL4 signaling **will limit metastasis** because of reduced cell survival in secondary sites.

Methods and Results:

Firstly, expression of the receptor for IL4 was genetically 'knocked down' in a pair of murine mammary tumor cell lines. We generated several clones of the 4T1 cell line that showed knockdown by 75-90% of the IL4 receptor. Two control clones had no change in IL4 receptor

level, as expected. The PyVT-R221A cell line was more recalcitrant, however we successfully generated a clone with 55% knockdown, in addition to controls.

Initial characterization of knockdown (KD) and control clones showed significantly slower growth rates in vitro as assessed both by metabolic assays and direct cell counting.

Additionally, control clones responded to increasing concentrations of exogenous recombinant IL4 with increased proliferation, but this response was not seen in the knockdown clones. Flow cytometric analysis using either annexin V staining or sub G1 measurement showed that clones lacking IL4 receptor had 2-fold higher basal levels of apoptosis.

To assess apoptosis and proliferation post-chemotherapy, we initially used metabolic (MTT) assays and varying doses of paclitaxel. 4T1 cells are sensitive, but not at low doses. By MTT assay, a dose of 5 mM paclitaxel was identified as the IC50 for the control clones, however 2 of 3 of the KD clones required 10-20 mM to kill 50% of the cells. This was in the absence of exogenous IL4.

Under basal conditions, clonogenic survival was similar amongst control and KD clones. When recombinant IL4 was added, there was an increase in colony size (indicating a proliferative effect) and colony number (indicating a survival effect) in the control clones. When paclitaxel was used in the presence of IL4, control clones demonstrated significantly more colonies than did KD clones under the same conditions. Thus signaling through IL4 receptor appears to confer a survival advantage, both in the absence and presence of paclitaxel.

To test effects in vivo, we have performed experimental and spontaneous metastasis assays using clones of the 4T1 cell line. Both pulmonary and hepatic metastatic lesions were significantly decreased [2.4-fold lower] in mice injected with IL4 receptor knockdown clones compared to controls. There was no significant effect on the primary mammary tumors.

Conclusions:

Together our results suggest that IL4 signaling in tumor cells promotes survival both in the absence and presence of a chemotherapeutic drug. This survival effect correlates with increased metastasis. Thus, blocking IL4 may be a useful therapeutic approach for preventing metastatic spread, and drugs in development for asthma have the potential to impact breast cancer metastasis.

BIBLIOGRAPHY

No manuscripts to report at this time.