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13. SUPPLEMENTARY NOTES					
14. ABSTRACT In recent years, progress has been made in the development of immune-based therapy for cancer. Conceptually, these treatment strategies have the potential of harnessing the immune system to combat and eliminate cancer cells. One major obstacle to the success of immunotherapy in both human and animal studies is the development of immunologic tolerance in tumor-bearing hosts. Therefore, the immune system fails to recognize cancer cells as dangerous and actively suppresses anti-tumor immune responses. Identification of the underlying mechanisms and the critical players that drive tolerance to the tumor is critical to improve the therapeutic efficacy of immunotherapy. Recent data indicate that activin A, a small protein secreted by some immune cells and by breast cancer cells has immune regulatory functions that may play a key role in promoting escape of tumors from immune control. The proposed studies will test the hypothesis that activin A secreted by breast cancer cells plays a key role in suppressing antitumor immunity. The goals are to demonstrate the role of activin A produced by breast cancer cells in tumor growth and metastasis, and the potential therapeutic benefit of blocking activin A to increase the response to radiotherapy.					
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1 Introduction

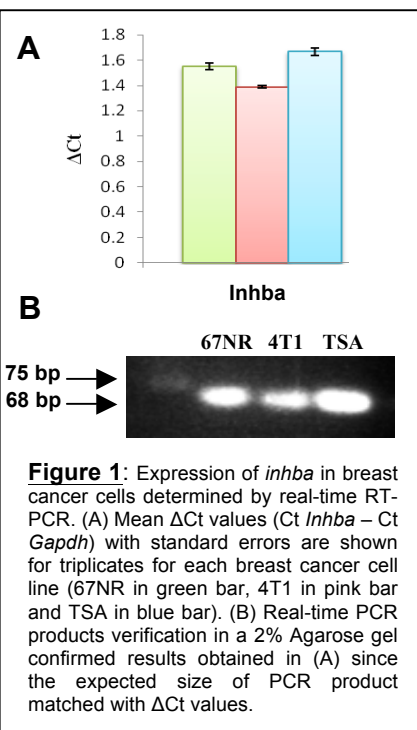
Owing to its ability to spread systematically, breast cancer remains a life-threatening tumor. Therefore, efforts in developing new treatment strategies are needed in order to eradicate metastatic breast cancer. In this respect, the activation of the immune system to elicit anti-tumor immune responses represents one of the most promising approaches that have recently demonstrated some success in other diseases. However, clinically apparent tumors have already harnessed host mechanisms to prevent immune activation and to induce an immunosuppressive microenvironment hindering immunotherapy-based treatments. As a consequence, the immune system fails to recognize cancer cells as dangerous and actively suppresses anti-tumor immune responses.

Identification of the underlying mechanisms and the critical players that drive tolerance to the tumor is critical to improve the therapeutic efficacy of immunotherapy. Recent data indicate that activin A, a small protein secreted by some immune cells and by breast cancer cells, has immune regulatory functions that may play a key role in promoting escape of tumors from immune control. The specific hypothesis of this project is that activin A secreted by breast cancer cells plays a key role in suppressing antitumor immunity. The goals are to demonstrate the role of activin A produced by breast cancer cells in tumor growth and metastasis, and the potential therapeutic benefit of blocking activin A to increase the response to radiotherapy (RT).

2 Body

NYU Langone Medical Center's main campus sustained significant damage during Hurricane Sandy, which struck NYU on October 29, 2012, affecting our patient care, research and education facilities. The Medical Center's main campus at 550 First Avenue started to reopen in November, and Tisch Hospital reopened its doors to patients in December 2012, however many labs and office spaces were relocated, and much equipment was irreparably damaged.

This caused considerable disruption and delay on our research. However, our laboratory has been relocated to a new facility, we have replaced the small animal irradiator critical for our work, with a dedicated preclinical irradiator XStrahl SARRP, and we now have available a state of the arts "mouse treatment core" on the same floor as the lab. With these resources in place, we were able to make rapid progress in the second part of the year.



In the first year, we proposed to investigate whether activin A produced by breast cancer cells contributes to generation of an immunosuppressive microenvironment *in vitro*.

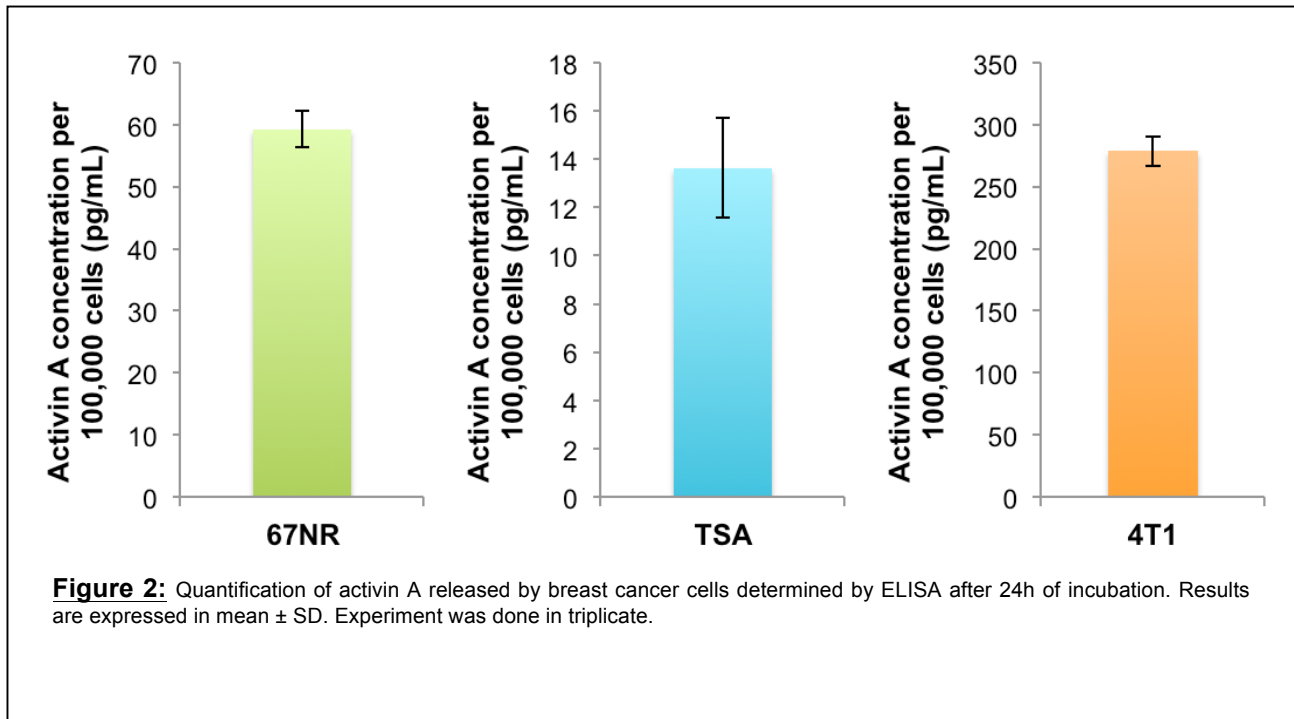
TASK 1: *In vitro* studies of activin A expression and function in breast cancer cells lines.

To determine whether breast cancer cell-derived activin A is a key modulator of immune cell function, we used three mouse breast cancer models, 4T1, TSA, and 67NR that represent tumors of different metastatic ability. 4T1 is poorly immunogenic and highly metastatic, spreading systemically by the hematogenous route from the primary subcutaneous site and causing death of mice within 30-40 days due to lung metastases [1]. TSA is poorly immunogenic and slowly metastasizes to the lungs, leading to gross metastases 60-80 days after initial injection at the primary subcutaneous site [2]. 67NR is not able to metastasize [3]. All three models are routinely used in our Lab.

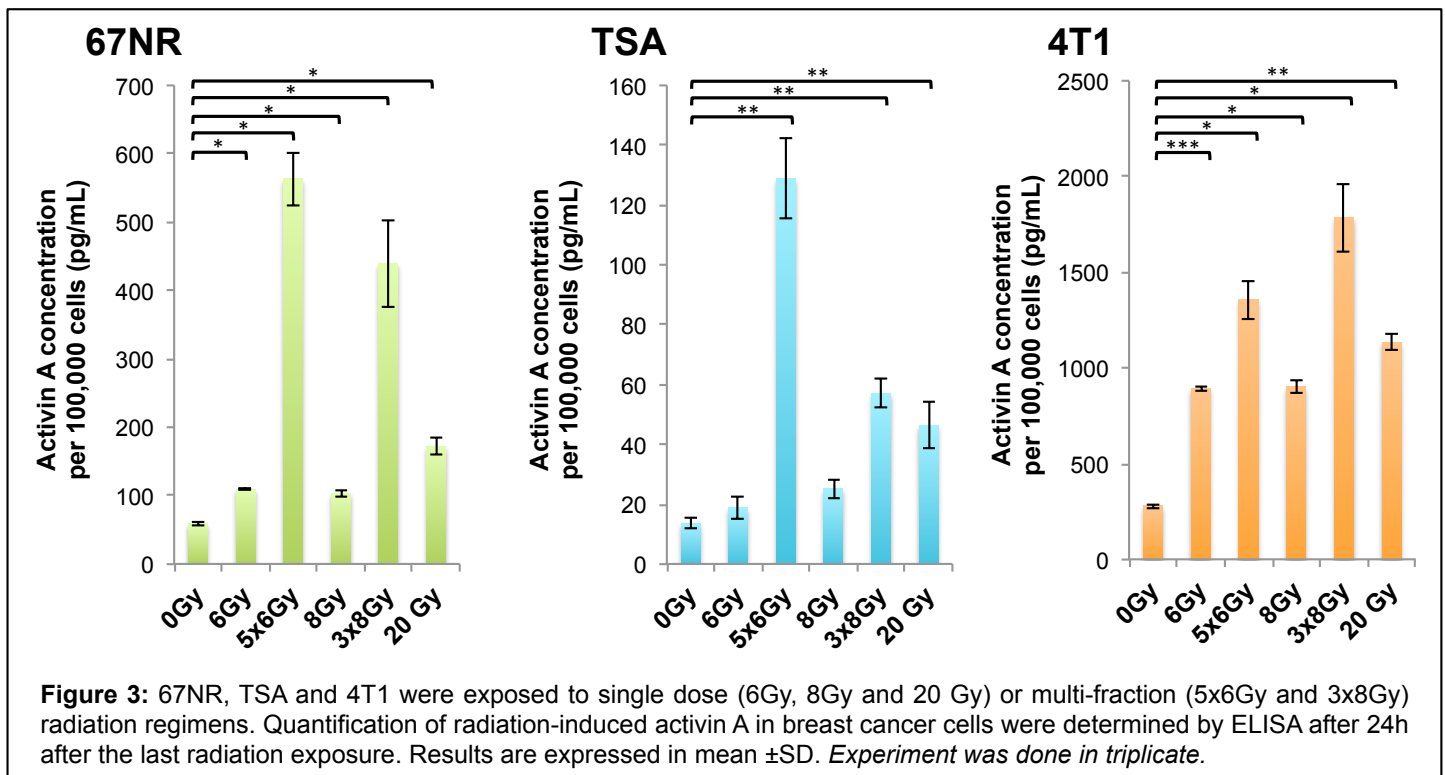
We previously showed that these breast cancer cells express *Inhba* gene encoding for activin A protein [4, 5] (Figure 1). Therefore, they provide a suitable system to test the role of activin A on tumor growth, metastasis and

Task 1.a: Quantification of activin A secretion by breast cancer cells (4T1, TSA and 67NR).

We first determined if activin A is secreted by 4T1, TSA and 67NR cells *in vitro* to confirm that *Inhba* gene is not only transcribed but also translated into protein. Results confirm that **all breast cancer cell lines are producing activin A** (Figure 2). However, even if breast cancer cells express comparable levels of *Inhba* mRNA, **only the most aggressive and metastatic 4T1 cells produced high levels of activin A** (67NR: 59.32 ± 2.91 ; TSA: 13.64 ± 2.06 and 4T1: 278.82 ± 11.95 pg/mL for 100,000 cells).



To investigate whether radiotherapy (RT) modulates the secretion of activin A by breast cancer cells, we exposed 4T1, TSA and 67NR to a single dose (6Gy, 8Gy and 20Gy) or multiple fractions (5x6Gy and 3x8Gy) of ionizing radiation. Irradiated cells we then plated for 24h after end of RT to quantify the release of activin A in the supernatants by ELISA. Results revealed that RT significantly increase the production of activin A by all breast cancer cells, more effectively after multi-fraction radiation regimens with an increase up to 9.5 fold compared to the non-irradiated cells (Figure 3 – 5x6Gy 67NR: 563.31 ± 38.85 ; TSA: 128.82 ± 13.43 ; 4T1: 1355.26 ± 99.83 – 3x8Gy 67NR: 438.79 ± 63.18 ; TSA: 57.04 ± 4.68 ; 4T1: 1784.45 ± 179.84 pg/mL for 100,000 cells).



These data indicate that **breast cancer cells increase activin A production in response to RT especially after a fractionated regimen.**

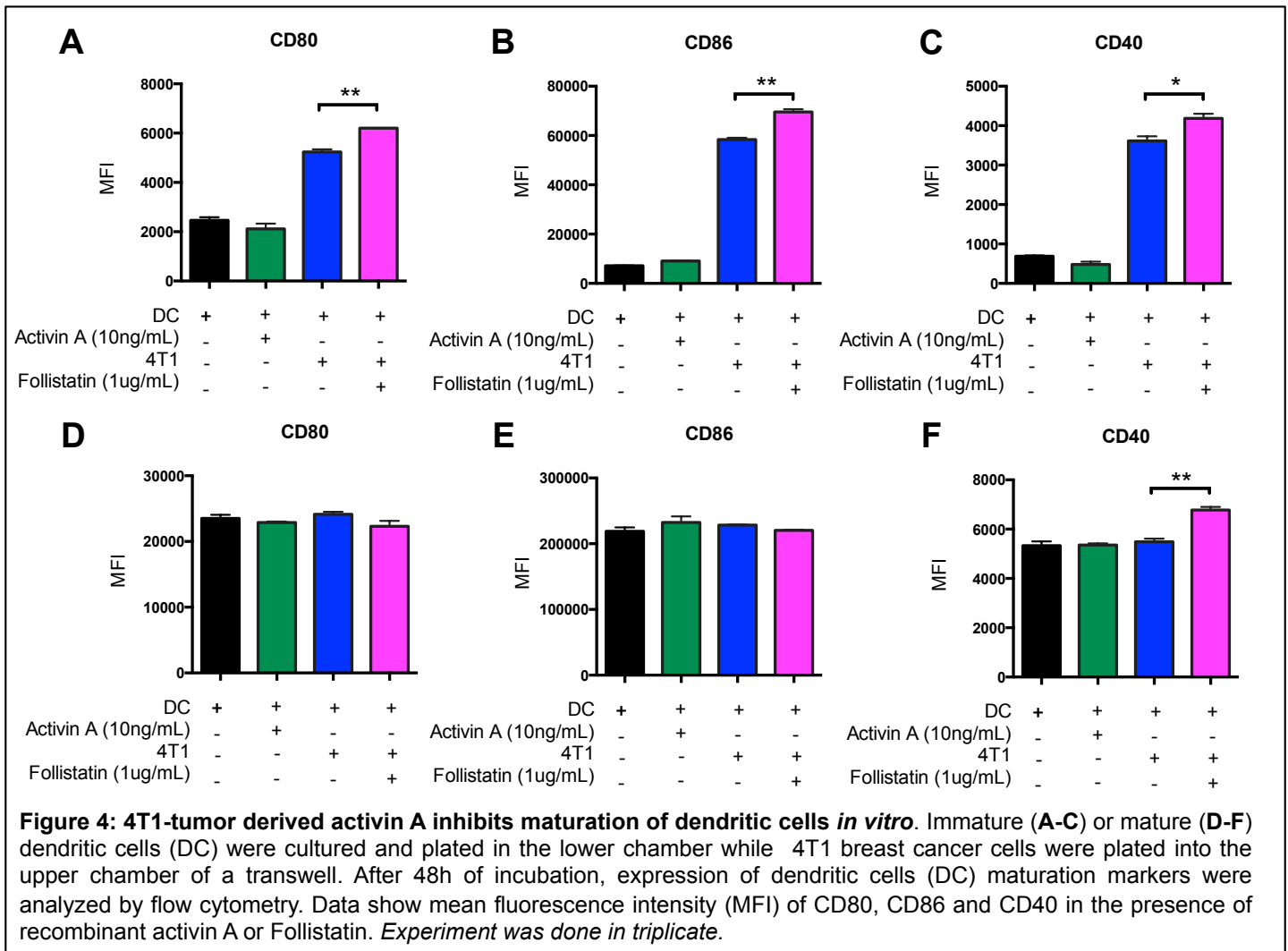
Task 1.b: Study of the effect of activin-A secreted by breast cancer cells on dendritic cells (DC) maturation.

Recently, it has been shown that activin A exhibit a potent autocrine effects on the capacity of human dendritic cells (DC) to stimulate immune responses. Indeed, it has been described that activin A secreted by human monocytes derived DCs and CD1c⁺ myeloid DCs inhibit their maturation [6, 7]. This suggests that tumor-derived activin A could promote immune suppression by precluding DCs maturation thus favoring a tolerance-inducing phenotype.

To test this hypothesis, we used a transwell system to analyze the ability of tumor-derived activin A to affect DC maturation *in vitro*. We tested the effect of activin A produced by 4T1 breast cancer cells because the latter produced the highest level of activin A (Figure 2). Immature DCs were plated into the lower chamber while 4T1 cells were plated into the upper chamber. After 48h of co-culture, expressions of DC maturation markers (CD80, CD86 and CD40) were assessed by flow cytometry analysis.

Data showed a significant increase in expression of CD80, CD86 and CD40 on DC co-cultured with 4T1, suggesting that tumor cells induce DC maturation, likely due to production of pro-inflammatory factors. Importantly DC activation was enhanced in the presence of the inhibitor of activin A follistatin (FS) (Figures 4A, 4B and 4C), suggesting that activin A secreted by the tumor cells limits the induction of DC maturation/activation. This supports our hypothesis hereby **tumor-derived activin A promote an immunosuppressive microenvironment by eliciting a tolerance-inducing phenotype of the DCs.**

To better understand the role of activin A on DCs, we asked whether tumor-derived activin A was able to reverse DC activation *in vitro*. Therefore, we repeated the previous experiment using DCs previously matured by exposure to LPS. As shown in Figures 4D, 4E and 4F, the high levels of costimulatory molecules and CD40 were not altered by culture with tumor cells or addition of recombinant activin A.



It has been demonstrated that RT can convert a tumor into an *in situ* vaccine by inducing an immunogenic tumor cell death promoting the cross-presentation of tumor-derived antigens by DC [8]. Because breast cancer cells secreted higher amount of activin A after radiation exposure (Figure 3), we asked whether DC exposed to irradiated tumor cells would not upregulate costimulatory molecules.

To that end, we exposed 4T1 cells to a single dose of ionizing radiation (6Gy, 8Gy or 20Gy) prior to plating them in the upper chamber of a transwell while immature DCs were plated in the lower chamber of the hanger. Dendritic cell maturation markers expressions (CD80, CD86 and CD40) were then analyzed after 48h of co-culture by flow cytometry analysis.

Consistent with data obtained with non-irradiated 4T1 cells, DCs expressed higher levels of CD80, CD86 and CD40 when activin A is blocked with its inhibitor Follistatin (FS) confirming a role for activin A in inhibiting DC maturation (Figure 5). Importantly, blocking radiation-induced activin A lead the highest expression of the DCs maturation markers CD80, CD86 and CD40 (Figure 5), especially after irradiating 4T1 with a single dose of 8Gy and 20Gy (Figures 5D, 5E, 5F, 5G, 5H and 5I).

Because immature DC can induce T cells tolerance [9], these data suggest that tumor-derived activin A may contribute to the status of immune tolerance seen in breast cancer-bearing hosts. More importantly, it suggests that higher levels of **activin A produced by irradiated breast cancer cells may counter, at least in part, the pro-immunogenic effects of RT and reduce cross-priming of anti-tumor T cells.**

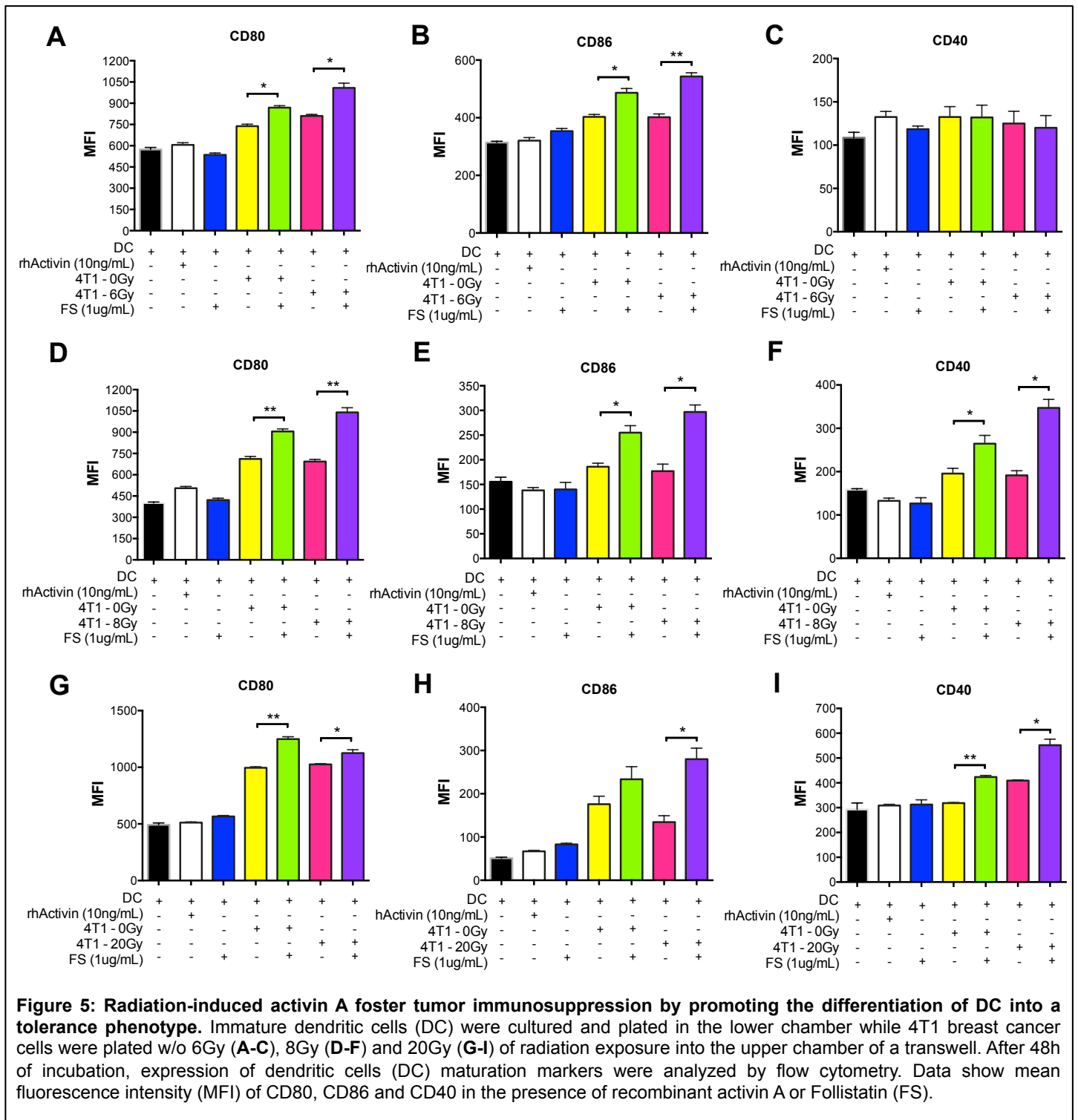


Figure 5: Radiation-induced activin A foster tumor immunosuppression by promoting the differentiation of DC into a tolerance phenotype. Immature dendritic cells (DC) were cultured and plated in the lower chamber while 4T1 breast cancer cells were plated w/o 6Gy (A-C), 8Gy (D-F) and 20Gy (G-I) of radiation exposure into the upper chamber of a transwell. After 48h of incubation, expression of dendritic cells (DC) maturation markers were analyzed by flow cytometry. Data show mean fluorescence intensity (MFI) of CD80, CD86 and CD40 in the presence of recombinant activin A or Follistatin (FS).

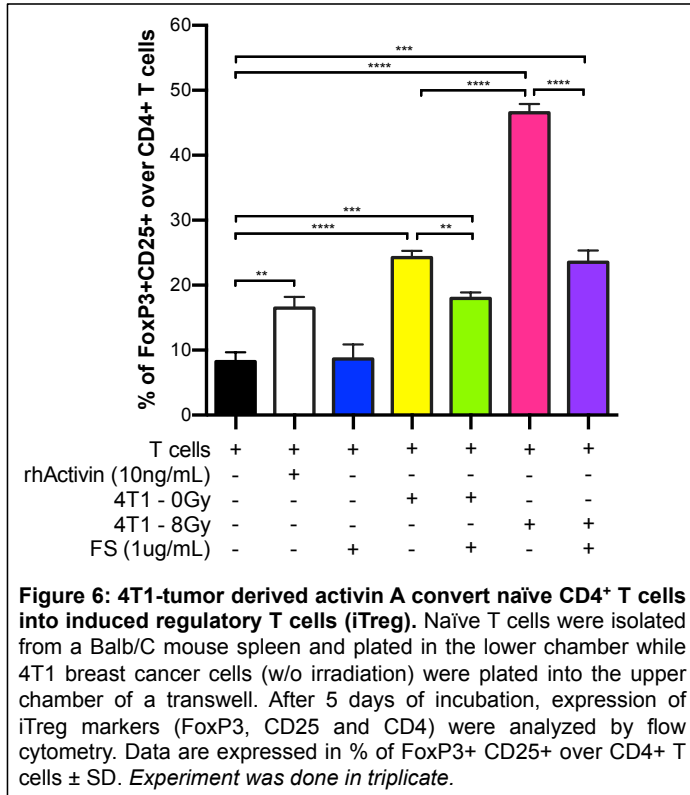
Task 1.b: Study of the ability of activin A produced by breast cancer cells to convert naïve CD4⁺ T cells into induced regulatory T cells.

In addition to its ability to inhibit the DC maturation, activin A has been shown to promote the TGFβ induced conversion of CD4⁺ T cells into FoxP3⁺ induced regulatory (iTreg) T cells [10]. To test the effects of activin A released by untreated and irradiated 4T1 cells on CD4⁺ T cells, we employed the same co-culture system. Naïve CD4 T cells isolated from a healthy mouse spleen, stimulated with anti-CD3 (1mg/mL) and anti-CD28 (0.5mg/mL) and placed into the lower chamber of the transwell. Non-irradiated or irradiated 4T1 (radiation dose: 8Gy) were then plated into the upper chamber of the hanger. After 5 days of incubation, the conversion of naïve CD4 T cells into iTregs (CD4⁺ CD25⁺ FoxP3⁺ cells) was evaluated by flow-cytometry. In line with literature, exogenous activin A lead to the conversion of 16.5% of naïve CD4⁺ T cells into iTregs versus 8.25% in control, while follistatin (FS) had no effect (Figure 6) [11]. Culture of T cells in the presence

of 4T1 cells led to a higher percentage of CD4 T cells differentiating into iTregs, which was decreased to 17.9% by blocking activin A with FS, suggesting that **tumor-derived activin A is partially responsible of the conversion of naïve CD4 T cells into iTregs.**

Since radiation enhances activin A secretion by 4T1 cells (Figure 3) we asked whether this increase in activin A could affect iTregs differentiation.

To test this hypothesis, we exposed 4T1 cells to a single dose of ionizing radiation (8Gy) before plating them in the upper chamber of a transwell while naïve CD4 T cells were plated in the lower chamber of the hanger. Data show that radiation exposure of breast cancer cells significantly promoted the conversion of iTregs with an increase in conversion up to 2-fold compared to non-irradiated 4T1 cells (46.6% of CD4⁺CD25⁺FoxP3⁺ compares to 24.2% for the non-irradiated 4T1; Figure 6). This effect was partially abrogated by follistatin (23.5% of iTregs) suggesting that **radiation-induced activin A fosters immunosuppression not only by reducing maturation of DCs but also by promoting the conversion of CD4⁺ T cells into iTregs.**



In the next funding period I will confirm the functionality of the iTregs using a suppression assay, and determine whether the radiation dose impact the conversion of naïve T cells into iTregs.

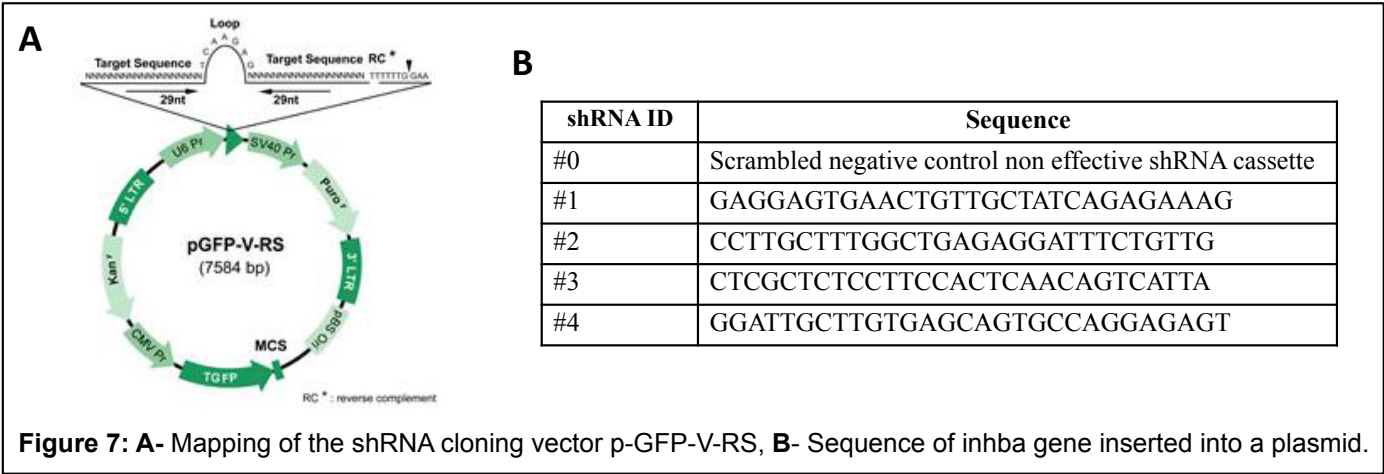
TASK 2: Effect of activin A blockade in vivo on breast cancer immunity.

To determine the role of tumor cell-secreted activin A *in vivo*, derivatives of 4T1 (4T1^{shInhba}) and TSA (TSA^{shInhba}) cells transduced with a set of plasmid encoding short-hairpin (shRNA) specific for murine *Inhba* have been prepared. Therefore, we began work on the Task 2.a of the specific aim 1.

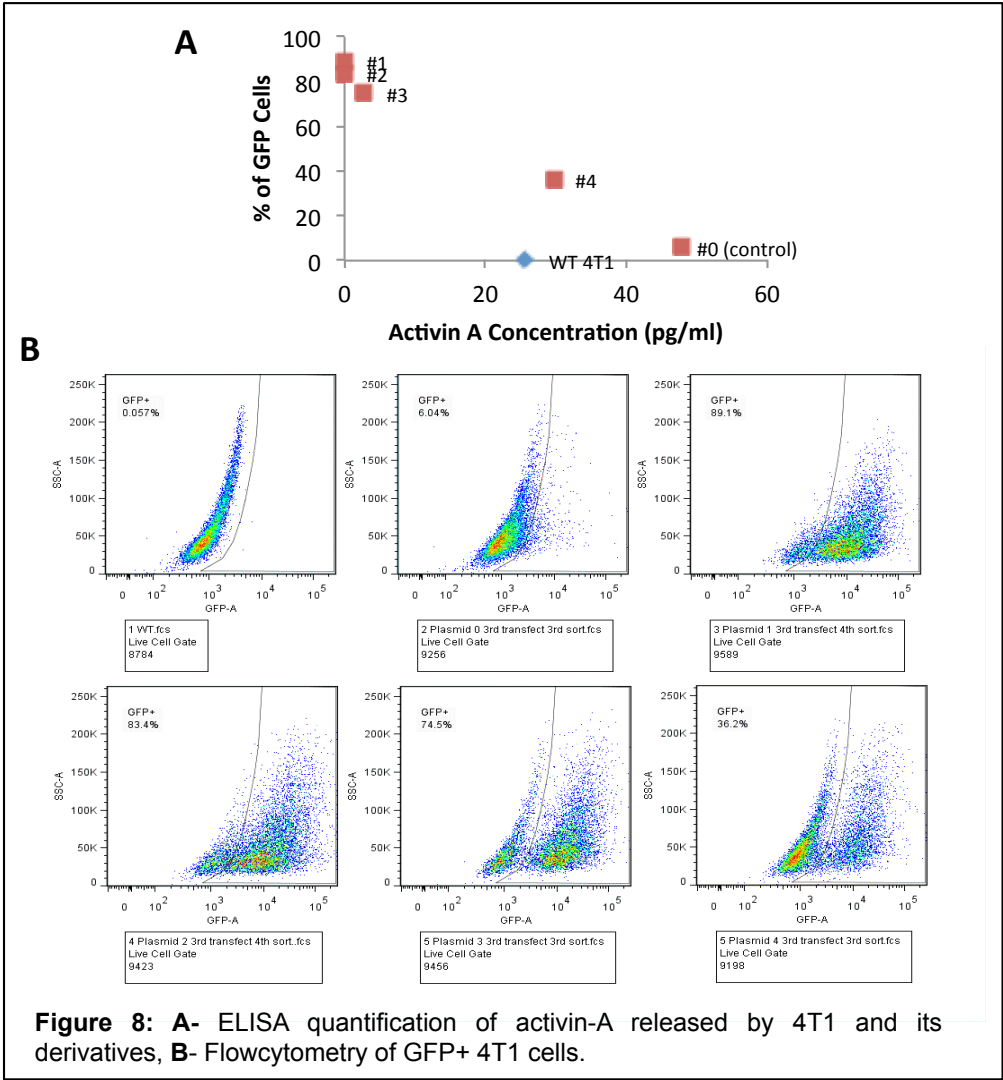
Task 2.a: Knockdown of 4T1 and TSA cell lines.

To knockdown *Inhba* gene in breast cancer cells, we first used a shRNA cloning vector p-GFP-V-RS from OriGene (Figure 7A). Because OriGene provides a set of shRNA constructs containing four different sequences of inhba gene-specific expression in p-GFP-V-RS plasmid (Figure 7B), we first determined which sequence was the best at knocking down *Inhba* gene. To that end, we established 4T1 cells with the five different GFP+ *inhba* shRNA plasmids according to the manufacturer's instruction. Wild type (WT) 4T1 or its derivative 4T1^{shInhba#1}, 4T1^{shInhba#2}, 4T1^{shInhba#3}, 4T1^{shInhba#4}, 4T1^{shInhba#0} were then we then plated to quantify the release of

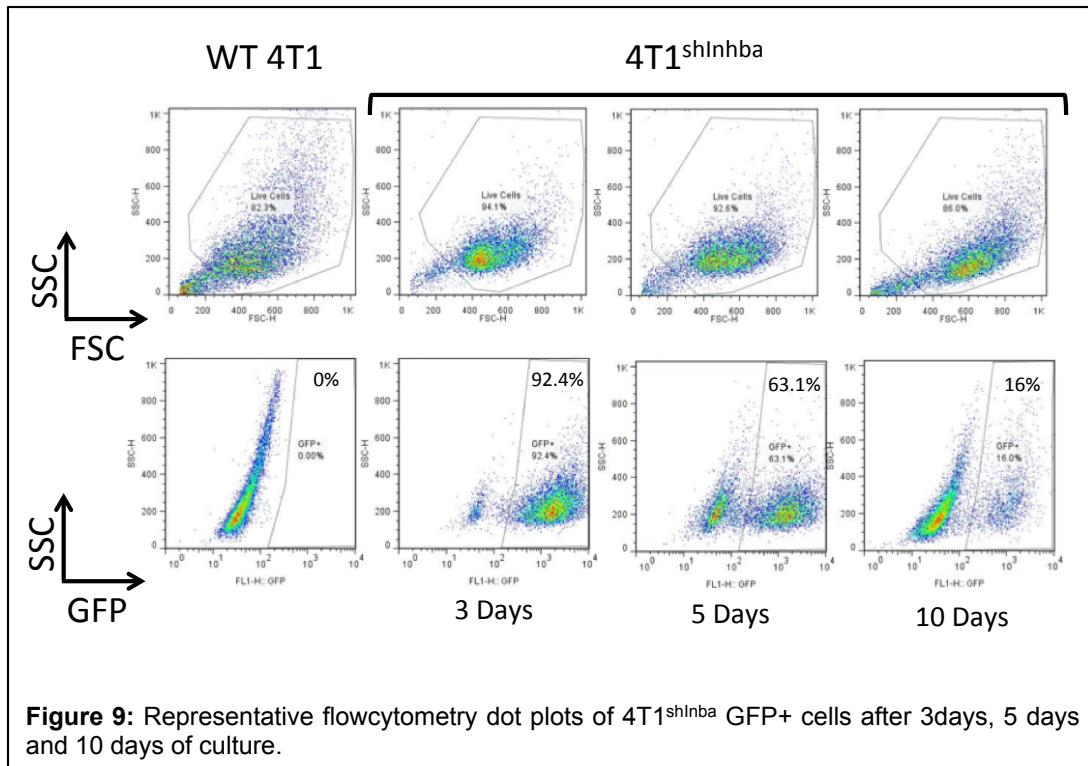
activin A in the supernatant by ELISA.



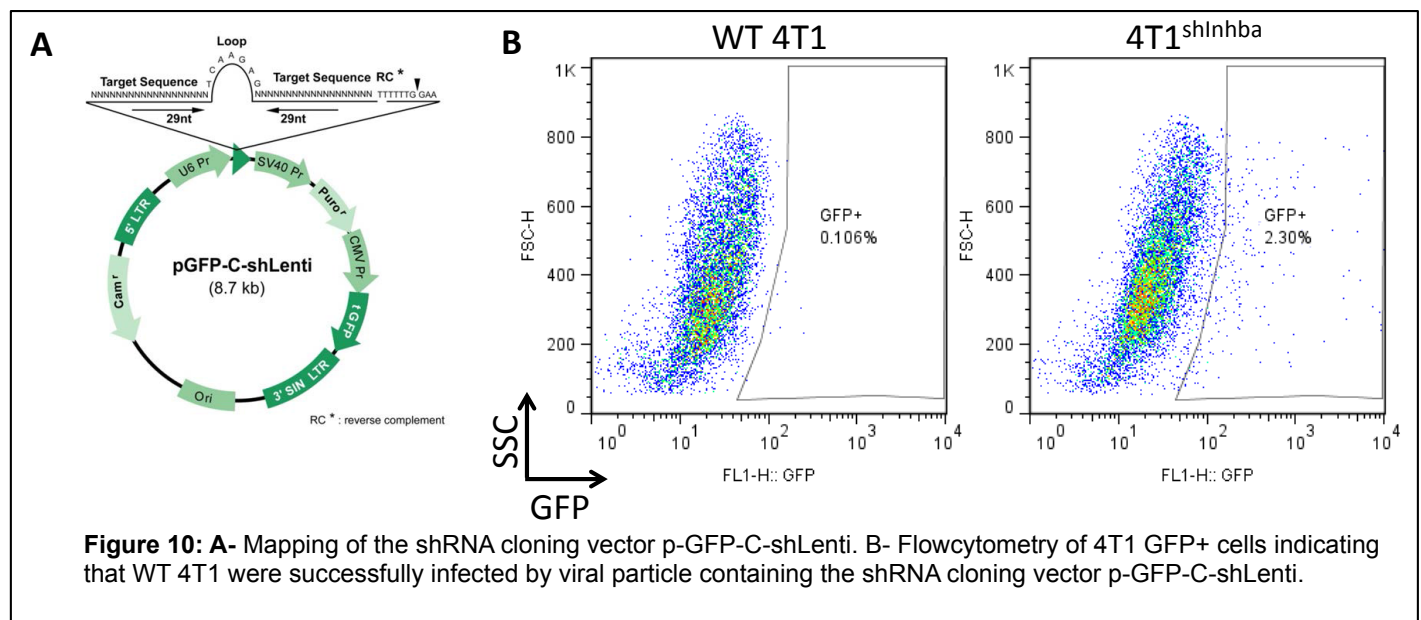
Results showed that shRNA carrying the sequences #1, #2 and #3 were the best at inhibiting activin A secretion by 4T1 (Figure 8).



Stability of the knockdown of activin A and control scrambled shRNA-transduced 4T1 cells *in vitro* were assessed by testing 4T1-derivatives for GFP expression after 3, 5 and 10 days of culture. As shown in Figure 9, 4T1 transduced cells gradually lost the GFP signal regardless of the shRNA used suggesting that the knockdown of activin A is not stable overtime.



As a consequence, we decided to change our approach by using lentiviral plasmids, well known to achieve a better stability compares to the p-GFP-V-RS ones. Therefore, another set of p-GFP-C-shLenti shRNA plasmids was purchase to OriGene (Figure 10A). By using 293FT packaging cells as well as an envelope plasmid, *pMD2.G*, and a packaging Plasmid, *psPAX2*, we were able to infect 4T1 cells with viral particle containing the p-GFP-C-Lenti shRNA (Figure 10B). The evaluation of activin A inhibition as well as the stability of the knockdown are currently under investigation.



3 Key Research Accomplishment

- Showed that breast cancer cells lines 67NR, TSA and 4T1 secrete activin A.

- Tumor-derived activin A fosters immunosuppression not only by impairing maturation of dendritic cells but also by increasing differentiation of naïve CD4 T cells into induced regulatory T cells.
- Increased secretion of activin A induced by radiation of breast tumor cells is functionally relevant since it further decreases DC maturation and enhances Treg generation, indicating that it may play an important role in reducing the pro-immunogenic effects of radiotherapy.

4 Reportable outcome

National Meeting and Presentations

- **2013 Radiation Research Society 59th annual meeting**

14-18 September 2013, New Orleans, LA.

Oral presentation:

Defining a molecular signature of pro-immunogenic radiotherapy in tumors.

John-Aryankalayil M, Vanpouille-Box C, Formenti S, Coleman N, Demaria S.

Poster:

Radiation-induced activin-A fosters tumor-mediated immunosuppression in breast cancer.

Vanpouille-Box C, Diamond J, Formenti F, Demaria S.

- **2013 Society for Immunotherapy of Cancer 28th Annual Meeting**

8-10 November 2013, New Harbor, MA.

Poster:

Radiation-induced activin-A fosters tumor-mediated immunosuppression in breast cancer.

Vanpouille-Box C, Diamond J, Formenti F, Demaria S.

Poster:

The abscopal effect of local radiotherapy is induced by TGF β blockade.

Diamond J, Vanpouille-Box C, Barcellos-Hoff MH, Formenti S, Demaria S.

Award

- Scholar-in-Training Travel Award, Radiation Research Society, 2013.

Institutional meetings and conferences

- NYU Immunology Club
Meets every Thursday 12PM
- NYU Cancer Institute Breast Biology Working Group
Meets every 3rd Wednesday of every month
- NYU Molecular Oncology and Tumor Immunology Works-in-Progress
Meets every Tuesday 5PM

- NYU Patho-Biology Works-in-Progress
Meets every Tuesday 6PM
- NYU Immunology and Inflammation Works-in-Progress
Meets every Wednesday 5:30PM

Mentoring

- Julie Diamond

Rotating student August 2012-December 2012.

Joined Sandra Demaria Lab after her rotation.

Collaboration

- Mary Helen Barcellos-Hoff, PhD.

Department of radiation oncology, NYU School of Medicine

We are closely collaborating with the lab of Pr Barcellos-Hoff on mechanisms of TGF-beta inhibition in cancer. Our collaboration has contributed significantly to our understanding of the immunosuppressive mechanisms within the tumor microenvironment by TGF-beta superfamily members.

Conclusion

Collectively, the *in vitro* data suggest that tumor-derived activin A contributes in generating immunosuppression by inducing a tolerogenic phenotype of the dendritic cells as well as enhancing conversion of naïve CD4 T cells into induced regulatory T cells. More importantly, similarly to TGF-beta, our results suggest that breast cancer cells upregulate activin A production in response to radiation exposure countering the pro-immunogenic effects of radiotherapy. As a consequence, breaking immune tolerance by inhibiting activin A in context of radiotherapy warrants further investigation. Since our data support a key role of activin A in immune tolerance by the tumor *in vitro*, we will be conducting experiments to confirm our finding *in vivo*. These experiments, as described in the approved statement of work, will comprise much of the work to be done in the second year.

In the past year, I have actively participated in departmental Works-in-Progress seminars and attended to NYU immunology club presentations as well as NYU Cancer Institute Breast Biology Working Group sessions, which have enriched my knowledge in cutting edge research in breast cancer. I have had the great opportunity to meet with leaders in the field of breast cancer immunology at recent meetings, and will continue to foster a collaborative relationship with them in the years to come. I continue to work closely with my mentor, Dr Sandra Demaria, who I meet with every week to discuss results and plan experiments. She continues to be an invaluable resource to my training as a future breast cancer scientist.

5 References

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6 Appendices

APPENDIX 1

(S701) Defining molecular biomarkers for pro-immunogenic radiotherapy in tumors.

Molykutty J. Aryankalayil, Claire Vanpouille-Box, Silvia Formenti, Norman Coleman, and Sandra Demaria.

APPENDIX 2

(PS4-57) Radiation-induced activin-a fosters tumor-mediated immunosuppression in breast cancer.

Claire Vanpouille-Box, Julie Diamond, Silvia Formenti and Sandra Demaria.

APPENDIX 3

Vanpouille-Box Claire, Diamond Julie, Siliva Formenti and Sandra Demaria. *Activin A is upregulated by radiation in breast cancer cells and promotes conversion of CD4 T cells into regulatory T cells*.

Journal for ImmunoTherapy of Cancer 2013 1(Suppl 1): P119.

APPENDIX 4

Diamond Julie, Vanpouille-Box Claire, Mary Helen Barcellos-Hoff and Sandra Demaria. *The abscopal effect of local radiotherapy is induced by TGFb blockade*.

Journal for ImmunoTherapy of Cancer 2013 1(Suppl 1):P115.

APPENDIX 1

(S701) Defining molecular biomarkers for pro-immunogenic radiotherapy in tumors.

Molykutty J. Aryankalayil, Claire Vanpouille-Box, Silvia Formenti, Norman Coleman, and Sandra Demaria.

ORAL PRESENTATION – 59th RRS annual Meeting.

(S701) Defining molecular biomarkers for pro-immunogenic radiotherapy in tumors.

Molykutty J. Aryankalayil¹; Claire Vanpouille-Box²; Silvia Formenti²; Norman Coleman¹; and Sandra Demaria²,

National Cancer Institute, Bethesda, MD¹ and New York University School of Medicine, New York, NY²

Local tumor radiotherapy (RT) can synergize with immunotherapy to induce anti-tumor immunity leading to tumor responses outside the irradiated field (abscopal effect). However, RT promotes pro-immunogenic as well as immune suppressive pathways: we hypothesize that some of these effects may depend on the dose/fractionation of radiation used. Our in vitro studies revealed that immune response pathways are induced by multifractionated (MF) but not single dose (SD) RT in human prostate cancer cells. Consistently, our in vivo studies in immunocompetent mice bearing syngeneic breast tumors showed that only MF (8 Gy x 3) but not SD (20 Gy) RT synergized with immunotherapy to activate an immune-mediated abscopal effect. Here we tested the hypothesis that SD and MF RT induce distinct changes in the tumor in vivo, allowing the identification of a gene signature relevant to proimmunogenic RT. To this end, we extracted RNA from TSA tumors growing in syngeneic BALB/c mice at 4, 24 and 48 h post-MF or-SD RT. Microarray analyses were performed using Agilent mouse mRNA Microarray Kit (V2) and data were analyzed using Ingenuity Pathway Analysis (IPA) and gene ontology classification. Over 100 immune response genes were differentially expressed (>2-fold, $p < 0.05$) in at least 1 of 4 comparisons (MF 4hr, SD 4 h, MF 24 h, SD 24 h). Importantly, IPA indicated that MF but not SD RT induces immune-related genes. Indeed, the 5 top canonical pathways upregulated at 4 and 24 h post MF RT included innate immune response, pattern recognition receptors implicated in sensing of PAMPS (pathogen-associated molecular pattern) and DAMPS (damage-associated molecular pattern), and interferon (IFN) signaling. Genes associated with IFN type I activation were significantly upregulated only in MF-treated tumors as early as 4 h and persisted at 24 h. Induction of interferon regulatory factor 7 (IRF7) in MF-treated tumors was confirmed by qPCR at 4h, 24h and 48 h. Overall, these results support a model whereby the pro-immunogenic effects of RT may require a multifractionated schedule and depends on activating genes commonly involved in the defense response to viral infections. We are currently testing this model in vivo. These experiments will provide insights into how to best harness ionizing radiation to optimize a combination with immunotherapy in the clinic.

APPENDIX 2

(PS4-57) Radiation-induced activin-a fosters tumor-mediated immunosuppression in breast cancer.

Claire Vanpouille-Box, Julie Diamond, Silvia Formenti and Sandra Demaria.

POSTER PRESENTATION – 59th RRS annual Meeting.

(PS4-57) Radiation-induced activin-a fosters tumor-mediated immunosuppression in breast cancer.

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Activin A is a member of the transforming growth factor beta (TGF β) superfamily and a pleiotropic cytokine that regulates many processes, from reproduction and development to cancer and immunity. Importantly, activin A shares the smad2/3 signal transduction pathway with TGF β and exhibits overlapping biological activities with the latter, including the ability to promote CD4⁺ T cell differentiation towards regulatory T cells (Treg). Recent data indicate that activin A is expressed by some tumors, including breast cancer. Radiotherapy (RT) has the ability to convert a tumor into an in situ vaccine, but the concomitant increase in Treg has been suggested to hinder its pro-immunogenic effects. Here we tested the hypothesis that activin A is upregulated by RT in breast cancer cells and contributes to generation of adaptive Treg in tumors. To this end, we employed three mouse breast cancer cell lines, 67NR, TSA and 4T1, which represent tumors of different metastatic ability. Expression of inhibin A (Inhba, the gene encoding activin A) was determined by qPCR. Secretion of activin A by untreated and irradiated tumor cells exposed to single dose (6Gy, 8Gy, 12Gy and 20Gy) or multifraction (5x6Gy; 3x8Gy) RT was quantified by ELISA. Transwell co-culture was used to assess the ability of activin A released by irradiated cancer cells to convert CD4⁺ T cells into Treg. While 67NR, TSA and 4T1 expressed comparable levels of Inhba mRNA, only 4T1 cells produced high levels of activin A (67NR: 37.1; TSA: 8.1; 4T1: 448.6 pg/mL for 10⁵ cells/24h). RT significantly increased activin A secretion, with the largest increase seen after 3x8Gy irradiation (67NR: 85.8; TSA: 55.1; 4T1: 993.1 pg/mL for 10⁵ cells/24h; p<0.05). Conversion of naïve CD4⁺ T cells into Treg upon activation in the presence of irradiated 4T1 cells was markedly enhanced (Control: 8.1%, irradiated 4T1: 46.9% of Treg). This effect was partially reversed in the presence the activin A inhibitor follistatin (23.8% of Treg). Data suggest that increased activin A secretion by breast cancer cells may contribute to the enhanced generation of Treg in irradiated tumors. Experiments are ongoing to determine whether targeting activin A will improve the pro-immunogenic effect of RT in vivo. Supported by DOD BCRP Post-doctoral fellowship W81XWH-13-1-0012.

APPENDIX 3

Vanpouille-Box Claire, Diamond Julie, Siliva Formenti and Sandra Demaria. *Activin A is upregulated by radiation in breast cancer cells and promotes conversion of CD4 T cells into regulatory T cells.*
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POSTER PRESENTATION

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Activin A is upregulated by radiation in breast cancer cells and promotes conversion of CD4 T cells into regulatory T cells

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From Society for Immunotherapy of Cancer 28th Annual Meeting
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Activin A is a member of the transforming growth factor beta (TGF β) superfamily and a pleiotropic cytokine that regulates many processes, from reproduction and development to cancer and immunity. Activin A shares the smad2/3 signal transduction pathway with TGF β and displays overlapping biological activities with the latter, including the ability to promote the differentiation of CD4 T cells to Th2 and regulatory T cells (Treg). Importantly, recent data indicate that activin A is expressed by some tumors, including breast cancer, suggesting that it could play a role in tumor escape from immune control. Radiotherapy (RT) delivered locally to a tumor induces the development of anti-tumor T cells, but its pro-immunogenic effects are hindered, in part, by concomitant activation of latent TGF β and increase of Treg. While different mechanisms have been implicated in RT-induced Treg increase, the pathways responsible for this effect remain unclear. Here we tested the hypothesis that activin A is upregulated by RT in breast cancer cells and contributes to the generation of adaptive Treg. Three mouse breast cancer cell lines, 67NR, TSA and 4T1, which represent tumors of decreasing immunogenicity and increasing metastatic ability, were used. Expression of inhibin A (Inhba, the gene encoding activin A) was determined by qPCR. Secretion of activin A by untreated and irradiated tumor cells exposed to single dose (6Gy, 8Gy, 12Gy and 20Gy) or multifraction (5x6Gy; 3x8Gy) RT was quantified by ELISA. Transwell co-culture was used to assess the ability of activin A released by irradiated cancer cells to convert naïve CD4 T cells into Treg. While 67NR, TSA and 4T1 cells expressed comparable levels of Inhba mRNA, only the most aggressive and

metastatic 4T1 cells produced high levels of activin A (67NR: 37.1; TSA: 8.1; 4T1: 448.6 pg/mL for 10⁵ cells/24h). RT significantly increased activin A secretion, with the largest increase seen after 3x8Gy RT regimen (67NR: 85.8; TSA: 55.1; 4T1: 993.1 pg/mL for 10⁵ cells/24h; $p < 0.05$). Conversion of naïve CD4⁺ T cells into Treg upon activation in the presence of irradiated 4T1 cells was markedly enhanced (Control: 8.1%, irradiated 4T1: 46.9% of Treg). This effect was partially reversed in the presence the activin A inhibitor follistatin (23.8% of Treg). Data suggest that increased activin A secretion by breast cancer cells may contribute to the enhanced generation of Treg in irradiated tumors. Experiments are ongoing to determine whether blocking activin A improves immune-mediated rejection of irradiated tumors in vivo. Supported by DOD BCRP Post-doctoral fellowship W81XWH-13-1-0012.

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APPENDIX 4

Diamond Julie, Vanpouille-Box Claire, Mary Helen Barcellos-Hoff and Sandra Demaria. *The abscopal effect of local radiotherapy is induced by TGF β blockade.*

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POSTER PRESENTATION

Open Access

The abscopal effect of local radiotherapy is induced by TGF β blockade

Julie Diamond^{1*}, Claire Vanpouille-Box¹, Mary Helen Barcellos-Hoff², Silvia C Formenti², Sandra Demaria¹

From Society for Immunotherapy of Cancer 28th Annual Meeting
National Harbor, MD, USA. 8-10 November 2013

Radiotherapy (RT) is employed to achieve local cancer control. However, in rare patients, regression of metastases outside of the radiation field has been reported after irradiation of one tumor site, a phenomenon known as abscopal effect. We have previously shown in experimental tumor models that the abscopal effect is mediated by activation of anti-tumor immune responses by radiotherapy, which can convert the irradiated tumor into an in situ vaccine. However, effective induction of anti-tumor immunity by radiation is rare. To study the barriers to the induction of an abscopal effect we have employed a mouse model of metastatic breast cancer. Ionizing radiation activates Transforming Growth Factor- β (TGF β), a strongly immunosuppressive cytokine that promotes DNA damage repair (DDR) and metastasis. We therefore hypothesized that neutralization of TGF β may improve the development of anti-tumor immune responses induced by RT, leading to an abscopal effect. To test this hypothesis, mouse mammary carcinoma TSA cells were injected s.c. at day 0 into syngeneic immunocompetent BALB/c mice at two separate sites, a "primary" site that was irradiated, and a secondary site outside of the radiation field. TGF β neutralizing 1D11 mAb was given i.p. starting one day before radiation. On day 12 when both tumors were palpable, mice were randomly assigned to groups receiving either 1D11 mAb or isotype control (MOPC-21) every other day for 16 days, with or without radiation (6 Gy doses given to the primary tumor on days 13-17). Radiation alone effectively delayed primary but not secondary tumor growth. 1D11 alone did not have a significant effect on either primary or secondary tumors. Combination of 1D11 with RT enhanced significantly inhibition of the primary irradiated tumor, with complete tumor regression in 4 out of 6 mice by day 28 ($p=0.0059$ radiation+1D11 versus radiation). Importantly, an abscopal effect was seen only in mice

treated with radiation + 1D11, with significantly smaller secondary tumors on day 28 ($p=0.0329$ radiation+1D11 versus MOPC-21). Data indicate that blocking TGF β in the context of radiation not only improves local tumor control, but also induces an abscopal effect with systemic tumor inhibition. Overall, data provide further support for the use of agents targeting TGF β during radiotherapy, a concept currently tested in a phase I/II clinical trial in metastatic breast cancer patients (NCT01401062).

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