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14. ABSTRACT

The origins of the invasive and metastatic phenotypes of breast carcinoma cells is a central unresolved question in cancer biology. Whereas some current ideas suggest that metastatic phenotypes are cell-autonomous lesions specified by the genomes of cancer cells, other views propose that metastatic traits are acquired through exposure of epithelial cells to paracrine signals from the tumor- associated microenvironment. **The major hypothesis in this DOD concept application is that phagocytosis of apoptotic breast cancer cells (BCCs) by tumor phagocytes will stimulate the production and secretion of immune modulating factors, chemokines, and angiogenic and metastatic factors in a paracrine manner that change the tumor microenvironment in favor of invasion and metastasis.** Although apoptotic cell phagocytosis (called efferocytosis) is often overlooked as a passive physiological event to clear unwanted cells, we hypothesize that clearance of apoptotic tumor cells by tumor phagocytes produce soluble factors that act as the principal priming signals for angiogenesis and metastasis.

15. SUBJECT TERMS
apoptotic cells, breast epithelial cells, immune modulatory factors, cytokines, phagocytosis

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Table of Contents

Introduction: The purpose of cancer chemotherapy and immunotherapy is to kill cancer cells, mostly by apoptosis. Phagocytes, which include macrophages and resistant tumor cells, will rapidly clear dying cells by recognizing molecular determinants on the apoptotic cell surface [1, 2]. Depending on how the tumor cells die, apoptotic cells can either elicit a non-inflammatory response towards phagocytes (called tolerogenic cell death) or an inflammatory response towards phagocytes (called immunogenic cell death) [3]. Because BCC themselves overexpress phagocytic receptors (such as αvβ5 integrin and uPAR) that stimulate their phagocytic activity an obvious question is to determine whether breast cancer cells, acting as tumor phagocytes, produce a skewed signature of immunosuppressive cytokines, chemokines, and growth factors that prime the tumor microenvironment for metastasis.

A major barrier to effective anti-cancer immunotherapy is the ability of the host to mount a durable anti-tumor response [4, 5]. To do so, the host must overcome intrinsic suppressive mechanisms that limit the sustenance of effective immune responses. It was with this background that we set out to explore whether breast cancer cells, acting in a phagocytic capacity, regulate immune suppression and tolerance.

Body: During the one-year award period, we proceeded with the experiments in the statement of work. A description of the progress of this study is described below.

Task #1: Set up MCF10A, MDA-231 and MDA-468 apoptotic-live cell co-cultures for factor profiling and chemotactic activity of conditioned media.

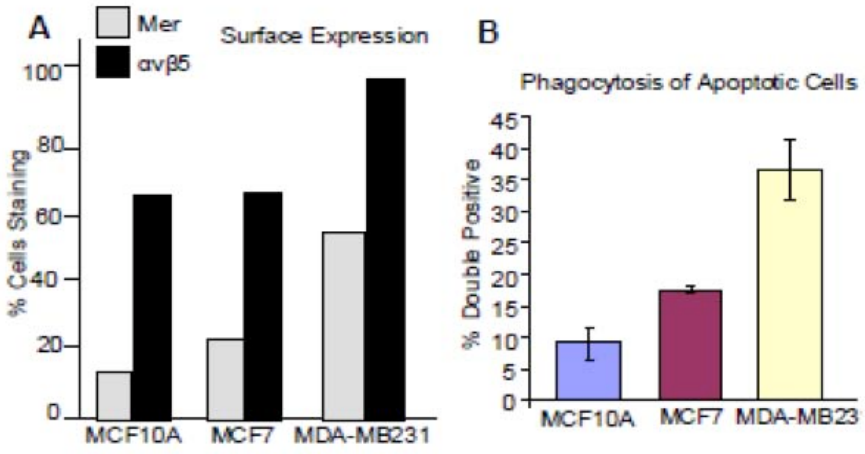

Fig. 1. Epithelial cells express receptors for the engulfment of apoptotic cells (A), and show elevated clearance of apoptotic Jurkat cells in tissue culture. In panel A, the surface expression of Mer and avb5 integrin, two well-known receptors that mediate engulfment were measure by FACS. In panel; B, engulfment was measured by a two-color FACS assay.

Experiment #1: Quantify phagocytosis by two-color FACS. We have successfully completed this task and shown in multiple experiments that MDA-MB-231 cells **(Fig. 1)** and MDA-MB-468 cells (not shown) have a significantly higher capacity for apoptotic cell engulfment compared to non-transformed immortalized MCF-10A and weakly transformed (ie non-invasive) T47D cells. We have also expanded the technology of two-color FACS analysis to use AMNIS Image stream methodology to quantify engulfment in breast cancer cells (**Fig. 2).** This improved technology adds 3-D resolution to confirm the fact that apoptotic blebs and bodies are internalized and not simply associated with the plasma membrane of the epithelial cells. Finally, we also used CypHer 5E loading to confirm that epithelial cells have specific phagocytic activity. CypHer5E is a pH-sensitive cyanine dye that accurately measures internalization. CypHer5E reports ligand-

mediated phagocytic internalization by exploiting pH differences that occur along the endocytic pathway.
Future studies are planned to monitor the trafficking itineraries in these cells to assess whether apoptotic material end up in the late endosomes.

Experiment #2: Analyze the levels of cytokines, growth factors, and chemokines by xMAP Bio-Plex Procarta cytokine arrays using a Luminex plat reader. We unfortunately have had great difficulty with the Luminex technology and do not have any definitive data to report from these efforts. Cell culture media from epithelial cultures showed very high background of tolerogenic cytokines and non-specific elevation of several chemokines in the array. There was no discernable increased using conditioned media from apoptotic cell epithelial co-cultures or apoptotic cell macrophage co-cultures (RAW264 cells). We suspect there exist non-specific expression patterns in the Luminex, as expression could not be confirmed by RT-PCR.

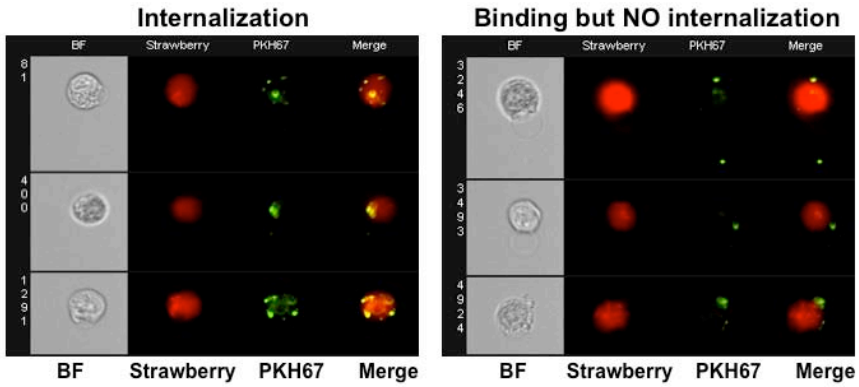

Fig. 2. Development of imaging technology to quantify clearance of apoptotic cells: Phagocytes were labeled red with rStrawbery and apoptotic cells labeled green with PKH67. Internalization (left) and binding can be observed using ImageStream.

Experiment #3: Quantify factors determined in task #2 (experiment #2) by RT-PCR. Given that the luminex technology was not carried out, we focused our attention on select immunosuppressive factors and chemoattractant factors, since this was the most relevant to the study design. Towards this goal, we examined IL-10, CCL2, and MIP1A but ELISA and IL-10 by a promoter-luciferase reporter assay (hIL-10 luc promoter) that was kindly provided by Dr. Karen Hedin (Mayo Clinic, MI) [6]. We have shown by both ELISA and luciferase promoter assays that IL-10 is upregulated during epithelial cell mediated phagocytosis of apoptotic cells compared to the conditioned media obtained from epithelial cultures in the absence of engulfment (**Fig. 3).** However, we have not observed increased IL-10 from MDA-MD-231 cells versus MCF-10A cells. The levels of CCL2 and MIP1A were not significantly increased during engulfment of apoptotic Jurkat cells.

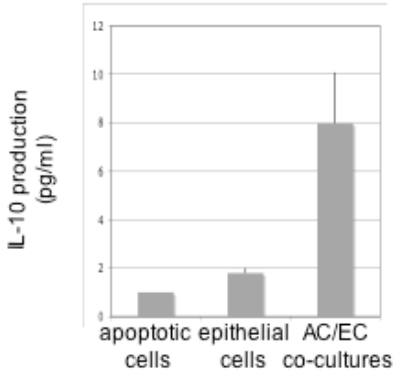

Fig. 3. Apoptotic cell (AC)/epithelial cell (EC)co-cultures produce immuno-suppressive IL-10. Either AC or EC were cultured for 24 hrs (bars 1 and 2), or were co-cultured for 24 hrs. IL-10 was examined by ELISA (above) or by luciferase (not shown).

Experiment #4: Determine chemotactic activity of conditioned media with purified monocytic pools. We next evaluated the ability of conditioned media from epithelial cells and epithelial/apoptotic

cell co-cultures to drive chemotaxis of RAW244.7 cells (macrophages) and DC2.4 (immature dendritic cells). Upper chambers were filled with 1.5% FBS + 50, 0000 cells while lower chambers were filled with CM + 1.5% FMS **(Fig. 4).** Interestingly, both epithelial CM and epithelial/apoptotic cell co-cultures caused migration through an 8 μm pore in the aforementioned assay conditions, suggesting that while epithelial CM is chemotactic, epithelial cells that had engulfed apoptotic cells did not have exaggerated activity.

Task #2: Characterize morphogenetic and apoptotic parameters in reconstituted 3-D matrigel cultures. Characterize infiltration of macrophages into spheroids.

Experiment #1: Optimize 3-D cultures in 3-D basement membranes. The 3-D matrigel cultures were set up and evaluated during the one-year DoD concept award. We used both MCF-10A cells and MDA-MB-231 cells to generate Matrigel spheroids, although luminal death and hollowing was mainly notable in the MCF-10A cultures. MDA-MB-231 cells did not form a hollow luminal cultures that is consistent with them being a highly transformed and invasiveness cells type. We have successfully set up the MCF-10A cultures in the 3-D models.

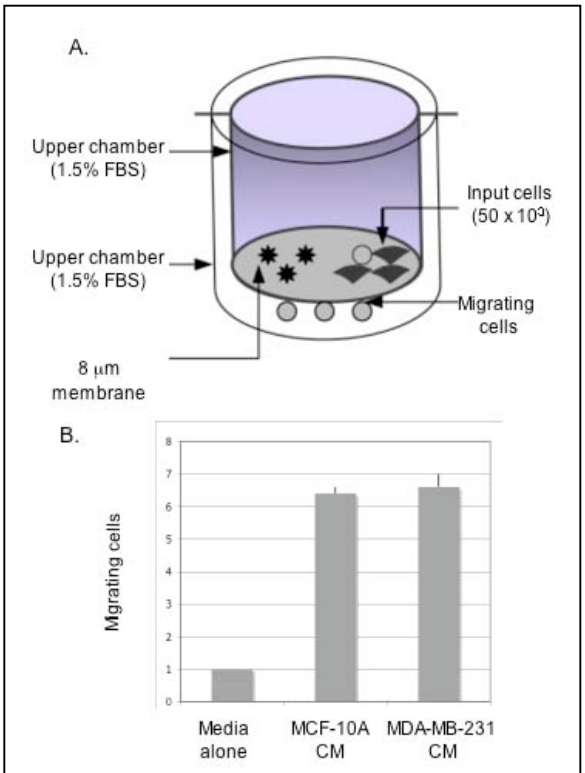

Fig. 4. Boyden chamber analysis to monitor chemo-attractant capacity of AC/EC co-cultures: Cells were cultured as in Fig. 3 except that CM was used in a boyden chamber

Experiment #2: Analyze cell death and phagocytosis in the 3-D matrigel cultures by microscopical and immunohistochemical methods. Since the hollowing of the lumen must be due to apoptosis and removal, we developed a microscopic analysis to quantify engulfment. Towards this goal, MCF-10A or MDA-MB-231 cells were labeled with 2 mM CypHer5E Mono NHS ester (GE Healthcare) in PBS for 20 min at RT, washed, and cultured in the 3-D matrigel for 1h at 14 days. After 14 days, the localization of apoptotic bodies from neutral cell surface (colorless) into acidic intracellular endosomes (deep red) was detected by excitation with a 635 nm laser with a Nikon fluorescence microscope. We identified over 4 times increase in fluorescence in the MCF-10A cultures compared to the MDA-MB-231 cultures. This suggests and that (i) epithelial cells are capable of engulfment bystander cells in the 3-D culture model, and (ii) that transformed cell have very delayed apoptosis. Ongoing studies will utilize the MCF-10 cultures forced to overexpress engulfment receptors that include TAMs and integrins.

Experiment #3: Analyze infiltration by macrophages and endothelial cells. Given the results of the experiments in task #1, experiment #4, we did not attempt these experiments.

We would like to emphasize that although the award period is completed, we will continue to pursue the role of epithelial tumor cells in tolerizing the tumor microenvironment, as outlined in

the original proposal. The one-year concept award has provided critical support to initiate this novel research project.

Key Research Accomplishments:

1. Showed that epithelial cells can act as potent phagocytes for apoptotic cells.
2. Showed that the more aggressive human breast cancer cells (MDA-MB-231 and T47D) have a higher phagocytic index over non-transformed immortalized MCF-10A cells.
3. Developed complementary methodology to quantify engulfment of apoptotic cells.
4. Demonstrated that epithelial cells, following engulfment of apoptotic cells, induce the production of IL-10 (including its secretion).
5. Showed that epithelial conditioned media from apoptotic cell/epithelial cells do not appear to produce chemo-attractant factors for macrophages and DCs.
6. Successfully generated 3-D breast cancer cultures for the exclusive analysis of engulfment of apoptotic cells.
7. Provided supportive evidence that tumor phagocytes may tolerize the tumor microenvironment by engulfing apoptotic cells.

Reportable outcomes: The experiments of the proposal are still in progress and no publications have resulted to date from these studies. We anticipate publishing these findings in the future.

Conclusions: Clinically, understanding the metastatic switches in tumor progression will have the greatest impact to reduce mortality from breast cancer. We hope these studies will provide rationale for new therapeutic targets that focus on tumor phagocytes as critical entities in breast cancer biology. The long-term goal will be to control or block phagocytic responses in order to prevent perverse alterations of the tumor microenvironment.

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