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Three-Dimensional Scaffolds to Evaluate Tumor Associated Fibroblast-Mediated Suppression of Breast Tumor Specific T Cells

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Abstract

In the tumor microenvironment, the signals from tumor-associated fibroblasts (TAF) that suppress antitumor immunity remain unclear. Here, we develop and investigate an *in vitro* three-dimensional (3D) scaffold model for the novel evaluation of TAF interaction with breast tumor cells and breast specific, neu antigen (p98) reactive T cells. Breast cancer cells seeded on 3D chitosan-alginate (CA) scaffolds showed productive growth and formed distinct tumor spheroids. Antigen specific p98 T cells, but not naïve T cells, bound significantly better to tumor cells on scaffolds. The p98 T cells induced potent tumor cell killing but T helper cell cytokine function was impaired in the presence of TAF co-seeding on scaffolds. We found that the immunosuppression was mediated, in part, by transforming growth factor beta (TGF- β) and interleukin-10 (IL-10). Therefore TAF appear capable of inducing potent T cell suppression. CA scaffolds can provide clinically relevant findings prior to preclinical testing of novel immunotherapies.

Keywords

breast cancer; immunotherapy; adoptive T cell therapy; IL-10; TGF- β ; tumor microenvironment

INTRODUCTION

During malignancy, changes in the tumor stroma (i.e., the nonmalignant component of the tumor that includes the extracellular matrix (ECM), vasculature, immune cells, and fibroblasts) are often associated with tumor growth, invasion, and metastasis.^{1, 2} The physical barrier formed by stromal proteins can prevent direct contact between tumor infiltrating immune effector cells (e.g., T cells) and malignant cells, while inducing production of transforming growth factor beta (TGF- β) that can directly block activation of immune cells and activate regulatory T cells (Tregs).^{1, 2} Stromal changes include the appearance of tumor-associated fibroblasts (TAF), which become differentiated from normal

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Supporting Information Available: RFP expression in stably transfected MMC-RFP line; morphology of MMC, fibroblast or T cell cultures grown on CA scaffolds or 2D culture plates, visualized by SEM; morphology of fibroblast cocultures grown on CA scaffolds or 2D culture plates, visualized by SEM. This material is available free of charge via the Internet at <http://pubs.acs.org>.

fibroblasts in a process driven by TGF- β and other cancer-derived cytokines.^{1, 2} Since TAF represents a major portion of the tumor stroma, they likely aid the tumor in immune modulation by impeding antitumor T cell function. TAFs, also known as activated fibroblasts, are thought to originate from epithelial-to-mesenchymal-transitioned cells. In addition to expressing markers (ER-TR7, vimentin, and fibroblast surface antigen of non-tumor derived fibroblasts), TAFs often express markers phenotypically associated with aggressiveness, including fibroblast activation protein, thrombospondin-1, stromelysin-1 and tenascin-c. Unlike non-tumor derived fibroblasts, TAFs also produce pro-tumorigenic growth factors (e.g., hepatocyte growth factor, epidermal growth factor, and interleukin-6) and factors associated with vascularization and metastasis (e.g., desmin, alpha-smooth muscle actin, and vascular endothelial growth factor).

Tumor specific T cells have the potential to eliminate antigen specific tumor cells; however, the inability of T cells to penetrate the tumor stroma currently represents a major hurdle to the success of anticancer immune therapies.^{3,4} In a recent breast cancer immunotherapy trial, the failure of an infused HER-2/*neu* specific CD8+ T cell clone to control tumor progression was largely due to tumor stromal barriers preventing penetration of T cells into malignant parenchyma.⁵ Histological analyses of advanced stage breast cancer and other cancers often reveal infiltrating lymphocytes trapped inside the tumor stroma.⁶ The presence of intratumoral immune cells directly correlates with improved survival of cancer patients,⁷⁻⁹ and has been recently associated with patient response to other therapies, including chemo- and radiation therapies. Understanding tumor-associated stromal contribution to cancer progression will improve our knowledge of growth promoting signals in the tumor microenvironment and lead to the development of new therapeutic interventions targeting the tumor stroma.

Malignant breast cells and stromal cells have been conventionally studied *in vitro* as 2D monolayers of cells; however, this has resulted in loss of 3D structure, which can negatively impact cellular interaction and function leading to discordant results. Moreover, methods based on xenografts in immunodeficient mice typically ignore the important contribution of the immune system. Therefore, the use of 3D culture systems will be beneficial for initial investigations of breast tumor/T cell interactions to bridge the gap between *in vitro* studies and preclinical testing in syngeneic and genetically engineered animals. The major advantage of 3D cell culture lies in the potential to restore cell-cell and cell-ECM signaling function and enables the assaying of malignant breast cells *in vitro* or *ex vivo* in a physiologically relevant microenvironment that more closely mimics the *in vivo* tumor architecture, tumor behavior, and signal transduction regulation.¹⁰⁻¹⁴

3D chitosan-alginate (CA) scaffolds represent an ideal scaffold system as these natural polymers have been demonstrated to be biocompatible and non immunogenic.^{10-12, 14-17} Both chitosan and alginate have the proxy structure of glycosaminoglycans (GAGs),²¹ a major component of the native extracellular matrix (ECM).²² Chitosan and alginate have been extensively used as biomaterials for tissue culture and regeneration and are FDA approved for various biomedical applications. In tissue engineering applications, CA scaffolds have been evaluated as a matrix for the deposition and stimulation of new bone tissue growth.^{16,17} The porous CA scaffolds could support feeder-free stem cell renewal¹⁵ and serve as an effective mimic of the tumor microenvironment for different cancer cell lines including glioblastoma, prostate cancer, and hepatocellular carcinoma.^{10-12, 14} Furthermore, the CA scaffolds are readily dissociated, enabling the release of cells for subsequent phenotypic and functional analyses.

While we have previously established that CA scaffolds are a suitable system for studying tumor cell growth and function, in this study we demonstrate that the CA scaffolds are a

suitable biomacromolecular complex for studying the interaction of three cell types. Co-seeding of tumor cells, T lymphocytes, and fibroblasts provided a breast tumor/immune microenvironment model for *in vitro* testing of the effect of tumor stromal cells on immunotherapy of breast cancer. The scaffolds provided an *in vitro* 3D tumor microenvironment ideal for productive growth of breast cancer cells and for the subsequent analysis of tumor/T cell interactions and tumor/T cell/fibroblast interactions. We assessed the physical interactions of the three cell types (MMC, fibroblasts, and p98 T cells) by SEM and confocal imaging, and investigated whether antitumor T cell function is impacted by TAF through evaluation of Th cytokine effector function by ELISA.

EXPERIMENTAL SECTION

Cell Cultures

Mouse mammary carcinoma (MMC) cells were maintained in 1X RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen), as previously described.¹⁸ Mouse neu p98-specific T cells were generated after immunizing neu-transgenic mice [FVB/N-TgN(MMTVneu)-202Mul] (without palpable tumors) s.c. 3 times (7-10 d apart) with 100 µg of neu peptide, p98-114 (RLRIVRGTLFEDKYAL) (Genemed Synthesis Inc., San Antonio, TX), as previously described.¹⁸ The single p98-114 peptide (RLRIVRGTLFEDKYAL) injection can induce CD4 and CD8 T cell immune responses because the peptide (MHC class II peptide) has an embedded MHC class I binding motif. Spleens were harvested and prepared as previously described 7–10 day post the last immunization.¹⁸ For p98 T cell expansion, splenocytes were seeded at 3×10^6 cells/ml in T₇₅ flasks. During the 21 day expansion, T cells were cultured in 1X RPMI medium supplemented with 10% FBS, 1% P/S, and 50 µM β-mercaptoethanol (Sigma-Aldrich Corp., St. Louis, MO), treated with IL-2 (recombinant human, 10 U/ml; Hoffman-La Roche, Nutley, NJ; days 4, 10 and 13) and IL-21 (recombinant murine, 100 ng/mL; PeproTech, Rocky Hill, NJ; days 10 and 13), and subjected to two rounds of *in vitro* p98 peptide (10 µg/ml) stimulations (day 0 and 9). T cells were activated on day 19 with soluble anti-CD3 antibody (50 ng/ml; eBioscience, San Diego, CA). For *in vitro* studies, T cells were used 2–5 days after anti-CD3 activation.

For co-culture studies, T cells were incubated with tumor cells and/or fibroblasts for 3 days. The 3-day time point was chosen based on previous characterization studies of the T cell line, which showed that maximal *in vitro* cytokine production by T cells occurred approximately 3 days after p98 peptide stimulation. Following peptide vaccination in female neu-Tg mice, generation of p98 T cells, and p98 peptide stimulation, the T cell line was characterized for functional CD4 and CD8 activity by enzyme-linked immunosorbent spot (ELISPOT) assay, as previously described.¹⁸

Generation of TAF

TAF were generated by digesting a spontaneous tumor of a Tg-MMTVneu mouse as described by Trimboli *et al.*¹⁹ In brief, a spontaneous tumor of a Tg-MMTVneu mouse was digested with 10 U/ml collagenase for 1 hour at 37°C. After two centrifugations at 200× g for 5 minutes, the supernatant (containing fat) was aspirated and the pellets washed with DMEM medium. The resuspended cells were plated in T25 flasks containing fully supplemented (10% FBS, 1% penicillin/streptomycin) DMEM medium. Cells were maintained in fully supplemented media and split as needed. To characterize the fibroblasts, the cells were detached by gentle scraping in PBS and plated into 6-well plates with coverslips at bottom of wells and stained (as described in the Phase Contrast and Immunofluorescence/Confocal Microscopy section) with ER-TR7, a fibroblast specific marker).

Fibroblast seeding on scaffolds were either done on same day as tumor cell seeding (same-day fibroblasts (fibro^s)) or on next day after T cells were seeded (next-day fibroblasts (fibroⁿ)). Unless noted otherwise, the T cell co-culture studies were performed with same-day seeded fibroblasts to allow greater adherence to scaffolds for the functional studies.

CA Scaffold Synthesis

3D 4% CA scaffolds were generated as previously described.¹⁰ In brief, scaffolds were prepared by mixing a 4 wt% chitosan solution with a 4 wt% alginate solution and cast in molds. The molds were incubated at 4°C for 12 hours, -20°C overnight, and lyophilized for 24 hours before sectioning into 2 mm thick, 13 mm diameter discs. The scaffolds were crosslinked with a 0.2 M CaCl₂ solution, washed in water, sterilized in 70% ethanol under vacuum, and then stored in PBS until use.

Cell Seeding on CA Scaffolds

MMC cells were seeded at 50,000 cells in 100 μ L complete media per sample, either directly in 12-well plate wells (2D) or on top of 3D CA scaffolds placed inside 12-well plates. The samples were incubated at 37°C and 5% CO₂ in a fully humidified incubator for 1.5 hours before 2 mL fully supplemented media was added to each well. Fibroblasts (250,000 cells) were either seeded at the same time as MMC cells (same day seeding) or 24 hours later (next day seeding). p98 T cells (5×10^6) were seeded onto scaffolds 24 hours after MMC cell seeding. Cells in 2D were dissociated using 1X Trypsin-EDTA solution (Invitrogen) and cells in 3D were dissociated from scaffolds using 1X Versene (Invitrogen), as previously described.^{12, 14}

Alamar Blue Assay

Proliferation of MMC cultured on 2D wells or CA scaffolds was evaluated by the Alamar Blue assay, according to the manufacturer's protocol. Briefly, prior to the assay cells on 2D or 3D were washed with PBS twice, and incubated with 1 mL of 10% Alamar Blue (110 μ g/mL Resazurin, Sigma-Aldrich) in fully supplemented DMEM at 37°C for 1.5 hours. The solution (300 μ L, per triplicate well) was transferred to a 96-well black-walled plate; absorbance values were obtained using a microplate reader at 540 nm. The cell number was derived based on standard curves.

Scanning Electron Microscopy (SEM)

Samples for SEM analysis were fixed as previously described.^{11, 12, 14} Briefly, samples were fixed with 2.5% glutaraldehyde in complete media for 30 minutes at 37°C. After fixing in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C overnight and dehydration in serial ethanol washes (0%, 30%, 50%, 70%, 85%, 95%, 100%), the samples were dried by critical point, sectioned, mounted, and sputter coated with platinum. Samples were imaged with a JSM-7000F SEM (JEOL, Tokyo, Japan). Bound naïve and p98 T cells were counted on ten random 2000 $\times$ images and the numbers of T cells per field of view were reported.

Phase Contrast and Immunofluorescence/Confocal Microscopy

MMC and fibroblast cell morphology was evaluated by phase contrast (live imaging) microscopy using a Nikon Eclipse TS100 inverted microscope (Nikon Instruments Inc., Melville, NY). For fluorescence immunostaining of fibroblasts, cells were stained for ER-TR7. ER-TR7 is an antigen located in the cytoplasm of reticular fibroblasts and a component of the ECM of both lymphoid and non-lymphoid organs.²⁰ The cells were first fixed with 3% formaldehyde and then stained with ER-TR7 rat monoclonal IgG_{2a} antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:100 in PBS at 4°C overnight, followed by incubation with Alexa Fluor 488 goat anti-rat IgG (Invitrogen) at 1:1000 in PBS for 1 hour

at room temperature. Cells were visualized at 100× magnification with a Nikon Eclipse TE2000-S inverted fluorescent microscope (Nikon Instruments Inc.).

For confocal fluorescence imaging of cells MMC-RFP cells (stable transfection) were seeded on CA scaffold samples and cultured for three days with p98 T cells, pre-labeled with Cell Tracker Green (Invitrogen), and fibroblasts. The scaffolds were fixed for 30 minutes in 4% formaldehyde in PBS, embedded in OCT cryo-compound (Fisher Scientific, Waltham, MA) in cryomolds (Fisher Scientific), and allowed to freeze on dry ice. The frozen blocks were cut into 15 µm sections and mounted onto glass slides. Slides were counterstained with 1 µM TO-PRO®-3 iodide (Invitrogen; nuclear stain) in PBS for 15 minutes, washed 3× in PBS for 10 minutes and mounted with VECTASHIELD® Mounting Medium (Fisher Scientific). Cells were imaged on a confocal laser scanning microscope (Zeiss 510 META LSM, Carl Zeiss AG, Oberkochen, Germany) at 630× magnification.

Flow Cytometry

MMC cells and fibroblasts were stained with mouse anti-neu antibody (anti-c-ErbB2/c-Neu (Ab-4) mouse monoclonal Ab (7.16.4); EMD Serono, Inc./Merck, Rockland, MA) in staining buffer for 20 minutes in the dark on ice, and then with an anti-mouse IgG FITC-conjugated antibody (eBioscience, San Diego, CA) for 20 minutes in the dark on ice. The samples were washed twice with washing buffer (PBS with 1% FCS), and resuspended in 500 µL of 4% formaldehyde in PBS. The samples were analyzed on a FACSCanto flow cytometer (BD Biosciences, San Jose, CA) and data was analyzed and plotted with the FlowJo software package (Tree Star Inc., Ashland, OR).

Live Dead Flow Assay

MMC cells were seeded on scaffolds in 12-well plates and the next-day incubated with increasing Effector:Target (i.e., Tc:MMC) ratios to assess the extent of T cell-mediated tumor cell death. Cells from all conditions were harvested from scaffolds as previously described and stained with a pacific blue dye (cytotoxicity marker) (Invitrogen), according to the manufacturer's instructions. The MMC cells were co-stained with mouse anti-neu antibody (anti-c-erbB2/c-neu (Ab-4); Calbiochem/EMD Chemicals Inc., Philadelphia, PA) in staining buffer for 20 minutes in the dark on ice, and then with an anti-mouse IgG FITC-conjugated secondary antibody for 20 minutes in the dark on ice. Cells were washed twice with washing buffer (1% FCS in PBS) and fixed in 500 µL of 4% formaldehyde in PBS. The samples were analyzed on a FACSCanto flow cytometer as described above. The cells were gated on neu+ cells to select the tumor cell population and then analyzed for the percentage of dead (i.e., pacific blue +) cells in each sample.

Enzyme-Linked Immunosorbent Assay (ELISA)

Media (1 mL) from the co-cultured cells were collected after three days in culture and stored at -80°C. Quantification of secreted murine cytokines (TNF-α, IL-10, or TGF-β) was performed by ELISA, according to the manufacturer's instructions for each cytokine (eBioscience). Experiments were repeated twice, with each sample assayed in a minimum of triplicate wells. Cytokine concentrations were reported in pg/mL.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA). The unpaired, two tailed Student's t-test was used to evaluate differences in cytokine production. A value of $p < 0.05$ was considered statistically significant.

RESULTS

MMC Tumor Cells Show Viable Growth on CA Scaffolds and Form Tumor Spheroids

We have previously shown the CA scaffolds to be a reliable model for replicating the natural ECM and tumor microenvironment of various cancers, including glioblastoma, prostate cancer, and hepatocellular carcinoma,^{11, 12, 14} and for promoting self-renewal properties of stem cells.¹⁵ Here, the ability of the CA scaffolds to provide a suitable growth environment for MMC breast cancer cells was assessed. The growth rates from seeding 50,000 MMC cells (standard seeding dose) and 100,000 cells showed similar growth kinetics, in both 2D and 3D conditions (Figure 1). Tumor cell number peaked after 10 days of seeding, in both 2D and 3D conditions, although the proliferation of MMC growth on CA scaffolds was comparably slower than 2D ($p < 0.05$). The slower proliferation has been attributed to a slower rate of diffusion of nutrients and oxygen to cells in the scaffold interior¹² and cell acclimation to their new environment. SEM revealed MMC cell incorporation and tumor spheroid formation in the CA scaffolds. After three days of culture, clusters of cells started to develop in the porous matrices of the CA scaffolds (Figure 2). By day 7 (and day 14), 3D tumor spheroids were clearly discernible. Tumor spheroids were not observed in 2D cultures (Figure 2).

TAF Isolated from Spontaneous Mammary Tumors of neu-Tg Mice Express Fibroblast Marker

We generated a *de novo* fibroblast line from the neu-Tg mice (see Materials and Methods). The fibroblasts were derived from a spontaneous tumor of a neu-Tg mouse and distinguished from MMC by microscopy, flow cytometry, and immunofluorescence. Compared to MMC cells (Figure 3a), the TAF showed the characteristic elongated, spindle-like morphology (Figure 3b). Few (0.45%) TAF stained positive for neu protein by flow cytometry (Figure 3c, right), but nearly 95% of MMC were positive, as expected (Figure 3c, left). Immunostaining of MMC (Figure 3d, left) and TAF (Figure 3d, right) with both a tumor specific marker, neu (red), and a fibroblast-specific marker, ER-TR7 (green), revealed expression of each marker only on each appropriate cell type indicating culture purity.

T cells and Fibroblasts Interact with MMC Spheroids

Images of fibroblasts, T cells, and MMC cells (MMC-RFP; Supplementary Figure 1) by SEM (Figure 4a-c, Supplementary Figure 2) and confocal microscopy (Figure 5) showed T cells with their characteristic uniformly round, small shape (approximately 2.5 μm in diameter) compared to fibroblasts (cell body approximately 10 μm) and MMC cells (heterogeneous in size, 6–15 μm in diameter) (Supplementary Figure 2a). When T cells alone (antigen-specific or naïve) were cultured with MMC on CA scaffolds, the antigen specific T cells, which are reactive to neu peptide p98-114 (RLRIVRGTLQFEDKYAL) of the HER/neu antigen, showed increased binding to the MMC spheroids than did naïve T cells (Figure 4a-b, top row). Indeed, quantification of bound T cells to spheroids revealed a significantly increased binding of p98 T cells compared to naïve T cells ($p = 0.0258$) (Figure 4d). When fibroblasts (Supplemental Figure 2b) and/or T cells (Supplemental Figure 2c) were co-cultured with MMC (Supplemental Figure 3), all cells on both 2D and 3D conditions were found localized near each other, as seen by SEM (Figure 4c). The close localization of the three cell types was also observed by confocal imaging (Figure 5). The tumor cells are indicated by RFP signal (Figure 5a) and T cells are shown by Cell Tracker green label (Figure 5b), with nuclear staining shown by the magenta signal (Figure 5c). The fibroblasts were left unstained and are the flat sheet-like cells seen at the right (and left) end of the cell cluster (Figure 5d), which show positive nuclear staining but lack green and red signals. All three cell types, therefore, appeared to co-localize within the scaffold cross-section.

p98 T cells are Capable of Inducing MMC Cell Death

The p98 polyclonal T cells comprise approximately 50% helper T (Th) cells and 50% cytotoxic T cells (CTL). These cells secrete high levels of Th1 cytokines. Regarding their cytotoxic function, the live dead flow assay revealed that there was no statistical difference in tumor cell kill at the highest Effector (T cell) to Target (MMC) ratio evaluated (100:1) compared to the lower ratios, following co-culture of MMC and p98 T cells in 3D CA scaffolds. Compared to untreated MMC (15% cell death), MMCs cultured with p98 T cells, at various E:T ratios, showed a significantly greater percent cell death (ranging from 67% to 89%; $p = 0.0005$ to $p = 0.0067$; Figure 6). As positive controls, T cells treated with phorbol-myristate-acetate and ionomycin (PMA/I) induced 60% cell death ($p = 0.003$ compared to untreated), and tumor cells subjected to freeze thaw (F/T) exhibited 94% cell death ($p = 0.0015$ compared to untreated). Therefore, p98 T cells demonstrated CTL activity against MMC at all E:T ratios tested. At the 100:1 ratio, there was approximately 75% cell death, significantly higher than in untreated MMC ($p = 0.0018$). We selected the 100:1 ratio as the E:T ratio for all co-culture studies for measurable functional activity in ELISA based on our prior experience.

Tumor Activated p98 T cells Secrete Less TNF- α in the Presence of Fibroblasts

Interferon γ (IFN γ) and Tumor necrosis factor α (TNF- α) is pro-inflammatory T cell cytokines secreted by Th1 during activation²¹ and Granzyme B is a protease secreted by activated CTL. No significant difference in IFN γ or Granzyme B secretion by T cells in the presence or absence of TAF was observed by ELISA assay. This might be due to the sensitivity of IFN γ or Granzyme B for the Eliza assay; a large number of cells are often needed for the detectability of IFN γ or Granzyme B secretion of T cells induced by stimuli. Therefore, TNF- α was used as a surrogate for Th1 function of the p98 T cells in the presence of fibroblasts. To determine if an earlier seeding of fibroblasts might result in a greater suppression of T cell function in the tumor scaffold environment, we studied T cell ability to secrete TNF- α in the presence of the next-day seeded fibroblasts (fibroⁿ) (Figure 7). In both 2D and 3D conditions, the fibroblast co-culture resulted in a significant decrease in production of TNF- α , compared to MMC + p98 T cells alone. The inhibition of TNF- α production by fibroⁿ in 3D (56%, $p = 0.0003$) is larger than that in 2D (47%, $p = 0.0008$) (Figure 7). Thus, the presence of TAF in tumor/T cell co-cultures could inhibit the cytokine effector function of tumor specific p98 T cells.

Immunosuppressive Cytokines, IL-10 and TGF- β , are Upregulated in Fibroblast Co-cultures

The inhibition of T cell activation by TAF might be associated with IL-10 and TGF- β production. Both IL-10 and TGF- β cytokines are prototypic mediators of immune suppression and are frequently upregulated in autoimmune diseases.²² Alone, tumor cells, p98 T cells or fibroblasts were found to secrete low levels of IL-10, however, only fibroblasts (not p98 T cells or tumor cells) were found to produce TGF- β (Figure 8). Fibroblast dose was assessed at fibroblast to MMC ratios ranging from 0:1 to 25:1. When fibroblast dose was increased, the levels of both IL-10 (Figure 8a) and TGF- β (Figure 8b) also increased accordingly.

DISCUSSION

In this study, we demonstrate the novel application of tumor associated fibroblast (TAF) seeded CA scaffolds as an *in vitro* model for investigating the impact of TAF on T cell function in breast cancer immunotherapy. We show that breast cancer spheroids can be established by direct seeding on 3D CA scaffolds. Growth of MMC cells in 3D was slow, unlike in 2D, as expected (Figure 1) and spheroid formation occurred as early as three days

after culture (Figure 2). As expected, tumor cell growth in 3D CA scaffolds was usually slower than on 2D plates. Studies showed that decreased proliferation actually more closely mimics the *in vivo* conditions than the rapidly proliferating cells on standard 2D cultures.^[9-11] The greater surface area of the spheroids (in 3D) appeared to facilitate enhanced tumor specific T cell binding, enabling maximal tumor-T cell interactions (Figure 4d). We have assessed tumor cell proliferation in the presence of tumor-associated fibroblasts and observed no apparent difference in tumor cell growth. As cell proliferation did not seem to be affected by the presence of fibroblasts (ECM source), anoikis (apoptosis of epithelial cells after detachment from extracellular matrix) may not occur in our system.

The ability to develop models or systems to rapidly study cell-cell interactions and cellular functions of singular components are invaluable. In this regard, we show the capability of CA scaffolds to assess interactions of individual cells (fibroblasts, p98 T cells, tumors) (Figures 4, 5) and the influence of one particular cell type on another (e.g., TAF on p98 T cells) with respect to breast cancer immunotherapy.

The presence of fibroblast did not appear to affect the T cell binding to MMC spheroids (Figure 5). In fact, in some instances, T cells seemed to associate more with fibroblasts than tumors in presence of fibroblasts (Figure 5). The impairment of T cell /MMC interaction may also be attributed to steric hindrance caused by T cell-fibroblast physical association. TAF have been reported in several cancer types, including lung and colon cancer, as a key culprit in tumor progression, immune escape, and metastasis.^{23, 24} The ability of TAF to influence tumor specific T cell function, even when isolated from other stromal cell components, underscores the important role of TAF in modulating the tumor microenvironment. In our study we demonstrate that we are able to isolate TAF, bearing fibroblast-specific markers (Figure 3), from mammary tumors of neu-transgenic mice and are able to maintain the cells in culture.

Our preliminary functional studies show that not only do TAF and p98 T cells interact with MMC spheroids, but TAF exert potent immune suppression. This was observed by an impairment of the p98 T cells in the presence of TAF, to secrete TNF- α during antigen recognition (Figure 7). The TNF- α secretion result (Figure 7) suggests a difference in T cell activity between 2-D and 3-D platforms. Such a difference may reflect a real biological difference due to a more physiological, “*in vivo*”-like environment of 3D CA scaffolds.

The presence of fibroblasts in the tumor milieu of the scaffolds appeared to be associated with increased IL-10 (Figure 8a) and TGF- β (Figure 8b) production. The increase in IL-10 and TGF- β demonstrated in fibroblast co-cultures is more likely a synergistic effect, as the amounts of IL-10 and TGF- β secreted by either fibroblasts, MMC or T cells alone are very low. It is known that TAF can secrete TGF- β , along with other pro-inflammatory cytokines, including IL-1, IL-6, and IL-23.²⁴ Moreover, the Th2-attracting chemokines and cytokines (such as IL-10) produced by TAF promote a Th2 type infiltration and inflammation in the tumor, which has been correlated with reduced patient survival.²⁵

Future studies into the functional characterization of TAF are needed, as well as comparison to non-tumor derived fibroblasts. Our study shows the utility of CA scaffolds as a model for characterizing tumor/immune cell interactions and lays the groundwork for future studies, including the precise roles of TGF- β and IL-10 in T cell inhibition. The CA scaffolds, provide a convenient and valuable *in vitro* model for studying clinically relevant improvements to breast cancer immunotherapy.

CONCLUSIONS

We have demonstrated the utility of CA scaffolds in providing an easy and suitable platform for the *in vitro* evaluation of tumor-stromal-T cell interactions. The simple platform allowed us to easily identify TAF in modulating immune response in a model of breast cancer. Therefore, strategies to inactivate TAF in breast tumor stroma may be beneficial to augment the efficacy of current adoptive T cell therapies for breast cancer. These CA scaffolds may provide a convenient model to bridge the gap between *in vitro* and pre-clinical testing of novel immunotherapies by allowing researchers to probe individual contributing cell types in a more relevant 3D tumor milieu.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Growth of MMC tumor cells in the CA scaffold environment. Proliferation of MMC breast cancer cells on CA scaffolds or 2D culture plates after 1, 3, 6, 9, and 15 days of cell culture, as determined by Alamar Blue viability assay. Cells were seeded at (a) 50,000 or (b) 100,000 cells. Data is representative of two experiments.

Figure 2. Morphology of MMC breast cancer cells grown on CA scaffolds or 2D culture plates, visualized by SEM. Shown are images of MMC on CA (top row) or 2D (bottom row), after (a) 3 days, (b) 7 days, or (c) 14 days of cell culture; 500 $\times$ magnification; scale bar, 20 μ m. Data is representative of two experiments.

Figure 3.

Morphology and specific staining of MMC and tumor associated fibroblasts (TAF). Brightfield phase contrast images of (a) MMC and (b) fibroblasts, 100 $\times$; scale bar, 10 μm . (c) Flow cytometry of neu expression on MMC (left panel) and fibroblasts (right panel). (d) Immunofluorescent co-staining neu (red) for MMC and ER-TR7 (green) for fibroblasts, with DAPI counterstain, 200 $\times$; scale bar, 10 μm . Data is representative of two experiments.

Figure 4.

Morphology of MMC/fibro/T cell cultures on CA scaffolds or 2D culture plates, visualized by SEM. Images of tumor cultures with: (a) naïve T cells, (b) p98 T cells, and (c) fibroblasts and p98 T cells cultured on CA (top row) or 2D (bottom row); 2000 $\times$ magnification, scale bar, 10 μ m. MMC: thick arrow, T cell: thin arrow, fibroblast: thin dashed arrow. Arrows denote representative cells. (d) Quantification of naïve T cells vs. p98 T cells bound to CA scaffolds (at 2000 $\times$ magnification from 10 different fields of view on SEM). Data is representative of three experiments.

Figure 5.

Confocal microscopy of MMC/fibro/Tcell cultures grown on CA scaffolds. Images from OCT-embedded scaffold sections of 3 day co-culture of: (a) MMC breast cancer cells (red; RFP), (b) p98 T cells (green; Cell Tracker dye) and fibroblasts (unstained); (c) nuclear counterstaining of cells (magenta; TO-PRO®-3 iodide dye); (d) brightfield phase contrast image; 630× magnification; scale bar, 10 μ m. Data is representative of two experiments.

Figure 6.

Evaluation of MMC tumor cell death after T cell co-culture by live dead flow assay. MMC/ T cell co-cultures were gated for: (a) the neu positive population (orange circle) and (b) excluded for CD3 expression. The gated neu positive cells were assessed for (c) percentage of dead (Pacific Blue positive) cells, shown by orange square marker. Histogram shown in (c) is of MMC untreated sample. (d) Shown are bar graphs representing mean percentage of dead cells of gated tumor population after T cell treatment at various effector to target (E:T) ratios (3:1 to 100:1); PMA/I: Phorbol-myristate-acetate and ionomycin; F/T: freeze thaw. Data is representative of two experiments. *Indicates a statistical difference with $p < 0.05$.

Figure 7.

TNF- α production by MMC-activated T cells in the presence of fibroblasts. MMC tumor cells were seeded on scaffolds; the next day fibroblasts (fibroⁿ) and T cells were co-seeded on scaffolds. Culture medium was assayed three days after T cell co-culture. Bars are mean and SE of triplicate wells, in pg/mL, for all conditions. Results shown are representative of two experiments.

Figure 8.

IL-10 and TGF- β production by MMC-activated p98 T cells in the presence of increasing fibroblast dose. Mammary tumor-derived fibroblasts were cultured with MMC in CA scaffolds on same day, and T cells added the following day. Culture medium was assayed three days after T cell co-culture for (a) IL-10 and (b) TGF- β secretion. Bars are mean and SE of triplicate wells, in pg/mL, for all conditions. Data is representative of two experiments.