**Metastatic breast carcinoma-associated fibroblasts have enhanced pro-tumorigenic properties related to increased IGF2 expression**

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No potential conflicts of interest were disclosed.
Abstract

Purpose: The microenvironment of metastatic breast cancer is incompletely characterized, despite prior evidence that it plays a key role in the biology of metastasis. A major component of the tumor stroma is the carcinoma-associated fibroblast (CAF), which has been shown to communicate with other stromal and cancer cells to create a pro-tumorigenic milieu. Our study was designed to characterize human CAFs from different metastatic sites.

Design: We collected 8 carcinoma-associated fibroblasts (mCAFs) from different metastatic sites and compared them to CAFs from primary tumors (pCAFs) and to normal breast fibroblasts (NFs). Molecular profiles and effects on breast cancer cell growth, on response to doxorubicin and on T cell proliferation were compared.

Results: We observed marked differences in mCAFs compared to pCAFs and NFs with respect to in vitro proliferation and effects on breast cancer cell migration, spheroid growth, invasion, response to doxorubicin and in vivo tumor growth. We found marked transcriptomic differences between mCAFs and pCAFs, including increased expression of interferon-related genes and IGF2 in the former. Cluster analysis revealed 2 groups of mCAFs, with the liver mCAFs clustering together, with increased pdgfa expression. Treatment with an antibody against insulin-like growth factors (BI836845) inhibited growth of mixed mCAF-tumor cell xenografts in vivo. Also, mCAFs had a suppressive effect on T cell proliferation.

Conclusions: This is the first comparative analysis of a set of CAFs from metastatic sites in breast cancer. It revealed a marked pro-tumorigenic effect in these mCAFs which occurs in part through increased expression of IGF2.

Translational Relevance
Chemotherapy resistance in metastatic breast cancer continues to be the foremost challenge in the treatment of breast cancer. Previous work focusing on the tumor cells themselves investigated the genetic and biological differences between the primary and metastatic breast cancer cells. In our study, we performed the first in depth molecular study of the most important cellular component in tumor stroma, carcinoma-associated fibroblasts, in metastatic breast tumor sites (mCAFs). We report a more aggressive, immunosuppressive and drug-resistant phenotype in mCAFs compared to primary tumor CAFs (pCAFs), associated with secretion of β-interferon and IGF2. Targeting IGF2 with a neutralizing antibody now in clinical trials (BI845836) inhibits these effects. These results provide a rationale for novel therapeutic avenues for the treatment of metastatic breast cancer and evidence for the more “pro-tumorigenic” and drug-resistant features of the tumor microenvironment in metastatic lesions.

Introduction
Metastatic breast tumors are almost always incurable: they progressively become resistant to chemotherapy as well as targeted therapies even if there is an initial response. The usual explanation for this inexorable process is selection of treatment-resistant clones, generated by the
inherent genomic instability of breast tumors. However, there is little clinical evidence for the selection of any “resistance gene” in metastatic breast cancers, even with Next Gen sequencing (1). The classic resistance gene, ABCB1 coding for P-glycoprotein, has not been clinically validated (2). One hypothesis to explain the incurability of metastatic lesions is that the tumor microenvironment protects tumor cells in metastatic lesions, more so than in the primary tumor. Moreover, this protection may vary from metastatic site to site, which may explain the frequent clinical observation of differential response to therapy in different metastatic sites. Much attention has centered on the essential interplay of the cells of the tumor microenvironment (such as fibroblasts, lymphocytes, macrophages, etc) in regulating the biology and progression of cancer cells. Extensive differences in tumor stroma compared to normal stroma have been widely observed and several studies have shown that carcinoma-associated fibroblasts (CAFs) may affect tumor cells’ sensitivity to cancer therapy. For instance, Straussman et al (3) showed that CAFs may cause drug resistance in melanomas via the secretion of hepatocyte growth factor (HGF). They also found that co-culture with CAFs rescued a HER2+ breast cancer cell line from sensitivity to lapatinib. However, this effect was not observed when cancer cells were treated with cytotoxic therapy. Although it is well established that CAFs from primary breast tumors (pCAFs) show significant molecular and phenotypic changes compared to normal fibroblasts (NFs), which confer on them a broad pro-tumorigenic ability, there have been almost no studies of CAFs obtained from metastatic sites and thus the contribution of metastatic CAFs (or mCAFs) to the incurability of metastasis is presently unknown. Indeed, there was previously no evidence that mCAFs are different from CAFs present within primary tumors. Through an ongoing metastatic tumor banking effort, we were able to isolate and grow 8 mCAFs from different metastatic breast tumor sites. Here we show that mCAFs show significant and marked phenotypic differences compared to both pCAFs and NFs. These differences are reflected in molecular differences especially regarding the expression of growth factors such as IGF2, β-interferon, which have clear therapeutic significance.

Materials and Methods

Antibodies, reagents and cell lines
Antibodies against total and phospho -Tyrosine, ERK (T202/Y204), Akt (S473) and STAT3 (Y705) were purchased from Cell Signaling Technology Inc. (CST, Beverly, MA). α-SMA, FAP, Actin, cytokeratin, vimentin, E-cadherin, N-cadherin and secondary antibodies were also from CST. The total and p-IGF1R antibodies and corresponding total IGF2 protein (CST, Beverly, MA) were a kind gift from Dr. Michael Pollak (McGill University). Recombinant Human IGF2 was obtained from Peprotech (Rocky Hill, NJ). Doxorubicin and paclitaxel were from Jewish General Hospital, McGill University (Montreal, Canada). Carboxyfluorescein succinimidyl ester (CFSE) were from StemCell technology (Ontario, Canada). Phorbol myristate acetate (PMA) and ionomycin were from Sigma-Aldrich (St. Louis, USA). LEAF purified anti-human CD3, CD3-PE and CD69-FITC were from Biolegend. Frozen PBMC (Human Peripheral Blood Mononuclear Cells) was from StemCell technology (Vancouver, Canada) with the health donor Lot# 1903040032. MDA-MB-436, BT-20, MDA-MB-231 and MCF-7 cell lines were purchased from American Type Culture Collection (ATCC). The cell lines were thawed for a maximum of 2 months experimental use, and then new vials were thawed. Periodically the mycoplasma was tested with MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza, Canada).
Isolation of CAFs
Primary and metastatic breast cancer tissue was obtained from breast cancer patients at the JGH in Montreal. The collection and use of human samples was approved by the Institutional Review Board (IRB), Jewish General Hospital (JGH, #05-006), which is in accordance with the Declaration of Helsinki and the Belmont Report. Patients signed informed consent to provide samples for the JGH Biobank for studies approved by the IRB of the JGH. pCAFs and NFs were described in (4) and mCAFs were generated with the same procedure. Normal, primary and metastatic CAFs tissue culture was carried out according to Hosein et al (4). All CAF experiments were performed within 2-10 passages after collection from tumor samples. After the isolation of CAFs from biopsies, mycoplasma testing was performed once the cell growth was stable, and then CAFs were frozen and stored in liquid nitrogen. For every new experiment, one frozen cell vial was used for two months in vitro culture. Periodically the culturing CAFs were tested for mycoplasma with a Mycoplasma Detection Kit (Lonza, Canada).

Generation of conditioned media (CM)
1x10^6 cells of each CAF were plated in a T75 tissue culture flask and cultured in 10 ml DMEM medium without FBS at 37 °C, 5% CO₂ in a humidified atmosphere incubator for 3 days. At day 3, the supernatant was collected and centrifuged to remove the cellular debris (1200rpm/min x 5 minutes). Cell-free conditioned media was then added to breast cancer cell line cultures for further experiments.

Cell migration and invasion assay
Transwell migration assay was performed in 6.5 mm diameter Boyden chambers with a pore size of 8.0 μm (Corning, NY). 5 x 10^4 MDA-MB-436 cells were suspended in 0.5% DMEM medium and placed in the upper compartment of transwell chambers and 700 μl of CM from the different CAFs was placed in the lower compartment. After incubation in a 37 °C, 5% CO₂ humidified atmosphere for 24 hours, cells in the lower surface of the top compartment were fixed in 4% formaldehyde for 20 minutes followed by staining with 0.05% crystal violet and five random fields of each were counted at 200x magnification. For invasion assays, cells were placed in 24-well matrigel-coated invasion chambers (Corning, NY). The lower chambers were filled with 700 μl CM from the distinct CAFs as a chemoattractant. A suspension of 5 x 10^4 MDA-MB-436 cells was plated in the upper compartment. The cells were incubated in a 37 °C, 5% CO₂ humidified atmosphere for 24 hours and then the membrane insert was fixed with 4% formaldehyde for 20 minutes and stained with 0.05% crystal violet, and stained cells counted.

Cell stimulation, lysate preparation, immunoprecipitation and immunoblotting
Breast cancer cell lines were incubated with CM from different CAFs (indirect co-culture), the CAFs themselves (direct co-culture) or with IGF2 (20ng/ml), then lysed in RIPA buffer (50mM HEPES, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) containing protease inhibitors. Cell lysates were centrifuged at 14,000 rpm, 4 °C for 20 minutes to remove cellular debris, then the supernatants were mixed with 6 x SDS sample buffer and boiled for 5 minutes. tumor samples from mice were homogenized in RIPA buffer containing protease inhibitors. For immunoprecipitation, tumor lysates containing 500μg protein were incubated overnight with 2μg of Ab and 35μl of 50% protein G-Sepharose (GE Healthcare Bio-Science Inc, Quebec, Canada). The immunoprecipitates were washed three times and boiled in 6 x SDS sample buffer. Following separation by SDS-PAGE and immunoblotting, proteins were
transferred to polyvinylidene difluoride membranes (Immobilon-p, Millipore, Etobicoke, ON) and immunoblotted with the indicated antibodies.

**AlamarBlue cell proliferation assay**

MDA-MB-436 cells were incubated with CM from the different CAFs for three days in 96 well plates. The supernatant was then replaced with 200ul 10% AlamarBlue for a three-hour incubation at 37 °C, 5% CO₂ humidified atmosphere. The resulting fluorescence was read on a FLUOstar Optima, using 560 nm (Excitation) and 590nm (Emission) filter settings and results were analyzed by plotting fluorescence intensity.

**GFP stable transfected MDA-MB-436 cells**

MDA-MB-436 cells were transfected with GFP plasmid for 48 hours, the cells were then trypsinized and 1 ml of complete DMEM medium was added. Plasmid containing clones were selected in 1mg/ml G418 supplemented medium and individual colonies were transferred to 12-well plate using cloning rings (Sigma-Aldrich, St. Louis, USA) for monoclonal expansion.

**Orthotopic breast cancer growth**

10⁶ or 0.7 x 10⁶ MDA-MB-436 cells were mixed with pCAFs (CAF53), mCAFs (BM113) and N-Fs (Norm2) at a ratio of 3:1 or 1:1, respectively, they were injected into the mammary fat pads of six-week-old female athymic nude mice (Charles River). Tumor growth was monitored twice a week and measurements taken with a digital vernier caliper. Mice with palpable (around 150-200 mm³) tumors were administrated via intraperitoneal injection with IgG isotype vehicle control antibody or IGF-2 blocking antibody BI836845 (100mg/kg) (Boehringer Ingelheim) twice/week for two weeks. Tumor volume (mm³) was determined with the formula (length × width²)/2. All the animal experiments were approved by the McGill University Animal Care Committee (Protocol Number: 2017-7931).

**Immunohistochemistry (IHC)**

Human samples were harvested and fixed in 10% neutral-buffered formalin (Fisher Scientific, MA, USA), processed, and embedded in paraffin. After deparaffinization and rehydration, 5 µM sections were prepared and antigenic epitopes were retrieved in Tris-EDTA buffer (pH 9.0) in a steamer. Sections were then incubated with primary anti-IGF2 antibody (Santa Cruz, gift from Dr. Pollak, McGill University) overnight at 4°C. The slides were then washed in PBS three times for 5 minutes and incubated with an anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) for one hour at RT. After addition of 3,3'-diaminobenzidine (DAB) according to manufacturer’s instructions (Sigma), the slides were sealed using VectaMount AQ Aqueous Mounting Medium (Cedarlane, Burlington, Canada).

**RNA extraction**

Total RNA from CAFs was extracted using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Ontario, Canada) following the manufacturer’s instructions; the quality and quantity of the total RNA were measured using an Agilent Model 2100 Bioanalyzer, and samples showing a RIN>9 were selected for further study. Samples were stored in RNase-free water at −80°C.

**Real-time polymerase chain reaction**
For RT-PCR analysis, total RNA was isolated from 10^6 cells, using a Qiagen kit. 1 μg total RNA was used for the first complementary strand generation using QuantiTect reverse transcription kit (Qiagen). The primers for gene expression analysis are as following: Human FAP (NM_004460): F- GATAACACTTACCTGCGTATG, R- ACAATCCCATGTCTGCGCAG; Human Nanog (NM_024865): F- GAATACCTCAGCCTCAGC, R- GCGTCACACCATGCTATTC; Human SNAIL (NM_178310): F- GCACCTCAGCCCAAGAA, R- GGACCACCCTCCTACCTG; Human Gapdh (NM_002046): F- AATCCCATCACCATCTTCCAG, R- TTCACACCCATGACGAACAT. All primers displayed 90% efficiency with a single melting curve in CFX-96™ Real-Time System (Bio-Rad, Mississauga). Expression levels of the housekeeping gene gapdh were used as controls.

**RNAi**

Validated siRNA targeting IGF1R (SI00017521 and SI02624552) and scramble control were purchased from Qiagen (Toronto, ON, Canada) and used according to the manufacturer’s instructions.

**Enzyme-linked immunosorbent assay (ELISA)**

To measure IGF2 production by different cells, 10,000 cells/well were seeded in a 96-well plate cultured alone or with the addition of 3,000 MDA-MB-436 or MCF-10 cells/well for 48 hours at 37 °C, 5% CO₂ humidified atmosphere with 10% DMEM medium. Then the cells were cultured in 200 μl DMEM without FBS for another three days and IGF2 levels measured using an ELISA kit (AnshLabs, TX) according to the manufacturer’s instructions. IFN-β and CXCL-12 levels were measured on the supernatants from the different cultured CAFs using the corresponding ELISA kit (R&D systems, Inc., Oakville, ON).

**Cancer stem cell detection**

MDA-MB-436 parent cells or incubated with the CM from the distinct CAFs were washed twice with phosphate-buffered saline (PBS) and then harvested. Detached cells were re-suspended in PBS supplemented with 0.5% FBS (1 x 10^6 cells/80μl). Fluorochrome-conjugated monoclonal antibodies against human CD44-FITC and CD24-AF647 (BD Bioscience, San Diego, California, USA) were added to the cell suspension as recommended by the manufacturer, and incubated at 4 in the dark for 30mins with swirling tubes every 15mins. The labeled cells were analyzed on a BD FACS Aria™ II.

**PBMCs isolated from whole blood, in vitro T cell stimulation and activation assay**

Whole blood was collected from healthy volunteer donors. Blood was then mixed with Ficoll-Paque PLUS (GE healthcare, Canada) and PBS (Phosphate-buffered saline) in a ratio of 1:1:1. The mixture was centrifuged at 400g for 35 mins at room temperature. The PBMCs at the interface were washed with PBS at 300g at 4°C twice and labeled with CFSE. We then stimulated them with PMA (50ng/ml) /IONOMYCIN (500ng/ml) for two or three days. PBMCs were also incubated in 96-well plate which was coated with ultra-LEAF purified anti-human CD3 antibody (10μg/ml) overnight for 2 days. At the end of the incubation, PBMC were stained with CD3-PE and CD69-FITC for T cell activation assay on BD FACS Aria™ II.

**Statistical analyses**
All statistical analyses were performed using the Student’s t-test with a 95% confidence interval with p < 0.05 interpreted as statistically significant.

**Results**

mCAFs have enhanced *in vitro* pro-tumorigenic and anti-cytotoxic effects on breast cancer cells.

Stromal fibroblasts were isolated from 16 patients from normal breast tissue or from primary or metastatic breast tumors, and designated NFs, pCAFs and mCAFs, respectively (Supplementary Table 1). We obtained 2 mCAFs from the same patient, one from a bone metastasis (BM69) and one from a liver metastasis (BM69L), the latter collected one year after the bone metastasis were collected. Each CAF sample showed strong vimentin and absent cytokeratin 18 staining (Supplementary Fig. S1A) as well as a mesenchymal cell shape. We observed that mCAFs displayed higher α-Smooth Muscle Actin (SMA) protein but similar fibroblast activation protein (FAP) protein expression compared to pCAFs and NFs (Supplementary Fig. S1B and S1C).

To examine how CAFs affect breast cancer cells, we chose triple negative breast cancer (TNBC) cell lines, MDA-MB-436 and BT-20, which represent the most aggressive form of breast cancer. (5). We found that conditioned media (CM) from mCAFs stimulated MDA-MB-436 cell proliferation significantly more than CM from pCAFs and NFs (Fig. 1A). Similarly, mCAFs induced stronger breast cancer cells MDA-MB-231 proliferation compared with pCAFs. Although the difference was less evident in MCF-7 cells (Supplementary Fig. S2A). CM from mCAFs also significantly increased the capacity of MDA-MB-436 and BT-20 cells to form colonies compared to CM from NFs and pCAFs (Fig. 1B). Moreover, compared with CM from NFs and pCAFs, CM from mCAFs induced significantly more migration and invasion in MDA-MB-436 cells tested in Boyden chambers (Fig. 1C and 1D).

In order to investigate the effects of direct co-culture of CAFs with TNBC cells, we grew CAFs as a feeder layer over which GFP-tagged TNBC cells were plated. The breast cancer cells formed spheroids when co-cultured over CAFs. We observed that breast cancer cell spheroids on mCAFs were significantly larger compared to those cultured over NFs and pCAFs (Fig. 1E). Taken together, these results suggest that mCAFs have greater pro-tumorigenic effects *in vitro* than pCAFs and normal fibroblasts.

As several studies have reported that CAFs are implicated in drug resistance (6-8), we studied the effects of the different CAFs on sensitivity to the chemotherapeutic drug doxorubicin in MDA-MB-436 breast cancer cells. We observed that CM from mCAFs did not significantly alter the sensitivity of MDA-MB-436 cells to doxorubicin, compared to NFs and pCAFs, although there was a trend towards a decreased sensitivity with CM from mCAFs compared to the NFs (p=0.08) (Supplementary Fig. S2). We then tested drug sensitivity in a 3-D model of co-culture spheroid growth using GFP-tagged MDA-MB-436 cells. GFP-MDA-MB-436 cells were mixed with stromal fibroblasts (in a 1:3 ratio) or grown alone on Matrigel and then treated with drug for 10 days. Doxorubicin treatment induced a significant decrease in 3-D growth of breast cancer cells in direct co-culture with NFs and pCAFs but not with mCAFs (Fig. 1F). These results suggest that mCAFs in direct contact with breast cancer cells offer greater protection from the cytotoxic effects of doxorubicin than pCAFs and NFs.

**mCAFs enhance breast cancer growth and metastasis *in vivo***

We next examined whether the distinct CAFs differentially support tumor formation *in vivo* using orthotopic mouse xenografts of MDA-MB-436 cells. Cells were mixed with human NFs
(Norm2), pCAFs (CAF53) or mCAFs (BM-113) at a 3:1 tumor cell:fibroblast ratio and then injected into the mammary fat pads of nude mice. We observed significantly larger tumors in mice co-injected with MDA-MB-436 and mCAFs compared to pCAFs and NFs (Fig. 2A and B). We then increased the relative number of fibroblasts to a ratio of 1:1 with tumor cells, maintaining the same number of total cells injected (1.3 x 10^6). As expected, owing to the decreased number of breast cancer cells, we observed that the emergence of the tumor was delayed (Fig. 2C). However, although the primary tumor size was comparable between the pCAFs and mCAF group but still significantly greater than the NF group, all mice injected with mCAFs displayed signs of metastatic disease, including ascites and intra-peritoneal tumor deposits (Fig. 2D and E, Supplementary Fig. S3A-S3C). Only one of 3 mice with a pCAF co-injection developed metastatic disease. Taken together, these results indicate that mCAFs are more potent in enabling tumor growth as well as metastasis than pCAFs and NFs.

**mCAF induced an EMT state in MDA-MB-436 cells in an in vitro co-culture model**

Epithelial-mesenchymal transition (EMT) is associated with the development of drug resistance, metastatic disease as well as stem-cell like properties (9). Breast cancer stem cells have been associated with a CD44highCD24low population. Approximately 10% of our MDA-MB-436 cells express these markers suggesting that a small sub-population of MDA-MB-436 cells may have properties of breast cancer stem cells. We found that incubation with CM from pCAFs modestly increased the CD44highCD24low population in MDA-MB-436 cells compared with NFs (Fig. 3A). However, CM from mCAFs induced a much greater CD44highCD24low population, so much so that this sub-population of cells constituted about 50% of MDA-MB-436 cells.

Cadherin switching from E-cadherin to N-cadherin expression is a critical cellular event in the process of EMT (10). We found that N-cadherin protein levels were increased while E-cadherin protein levels decreased in MDA-MB-436 cells directly co-cultured with mCAF, more so than with pCAF or NF co-cultures, when compared to levels in MDA-MB-436 cells alone (Fig. 3B). We also found that mCAF direct co-culture was more potent than direct co-culture with pCAFs or NFs in increasing RNA levels of SNAI1 and Nanog, two master regulators of EMT and stemness (11), in MDA-MB-436 cells (Fig. 3C and 3D). Taken together, these results indicate that mCAFs are more potent EMT and cancer stem cell inducers in MDA-MB-436 cells than pCAFs and NFs.

**The gene expression profile of mCAFs**

We next performed RNA-Seq analysis of all 16 fibroblasts grown in DMEM medium without FBS. Principal component analysis (PCA) revealed that pCAFs segregated with the NFs and away from the mCAFs (Fig. 4A). Moreover, the mCAFs segregated into 2 groups, one (cluster 1) with 4 mCAFs from skin, lung and bone metastases and the second (cluster 2) containing the 3 mCAFs from liver metastases and 1 from a bone metastatic site. Interestingly, the 2 mCAFs from the same patient segregated separately, with the liver mCAF together with the other liver mCAFs. Differential expression analysis was performed and we found that 331 genes were significantly up-regulated in mCAFs compared to pCAFs (>2-fold, p<0.05, >10 reads in all metCAFs) and 256 genes were down-regulated (<2-fold, p<0.05, >10 reads in all pCAFs). The top 10 genes over-expressed in mCAFs are shown in Supplementary Table 2. The most differentially expressed gene is IGF2 (insulin-like growth factor 2). A DAVID Gene Ontology analysis of up-regulated genes in mCAFs relative to pCAFs revealed that the biological
processes most significantly enriched in this gene set included interferon signalling, extracellular matrix (ECM) organization, response to hypoxia, and positive regulation of MAPK cascade (Fig. 4B). Ingenuity Pathway Analysis (IPA) analysis was performed on up and down regulated genes. The top 5 most significant canonical pathways include Hepatic fibrosis/Hepatic stellate cell activation and Interferon signalling. We then separately compared each of the 2 clusters of metCAFs to pCAFs and performed IPA analysis on significantly up and down regulated genes. We found that the most significant canonical pathway altered in non-liver mCAFs (cluster 1) is "Interferon signalling", while that in cluster 2 was "Hepatic fibrosis/Hepatic cell stellate activation", consistent with the predominance of liver metCAFs in this cluster. DAVID GO analysis of up-regulated genes in cluster 1 also identified interferon signalling as the most significantly enriched biological process (Supplementary Fig. S4A). We then measured IFN-β in the CM of these mCAFs using an ELISA assay and found that mean IFN-β levels were much higher in mCAFs compared to pCAFs and also 1.7 times higher in cluster 1 than in cluster 2 (p<0.01) (Fig. 4C). DAVID GO analysis of up-regulated genes in cluster 2 indicated an enrichment in genes related to inflammatory response, response to hypoxia and ERK signalling (Supplementary Fig. S4B). Levels of mRNA of PDGFA growth factor, one of the genes in the ERK signalling group, is expressed >2-fold higher in cluster 2 compared to the mean levels in cluster 1 mCAFs by both RNAseq and confirmed by qRT-PCR (Supplementary Fig. S5). In fact, RNAseq shows that PDGFA mRNA is expressed at a mean of > 2-fold higher levels in all mCAFs compared to all pCAFs (p<0.05). Differentially expressed genes between the 2 clusters were consistently differentially expressed in the same direction (liver vs bone) in the 2 samples from the same patient (BM69 liver and BM69 bone) (Supplementary Table 3).

mCAF derived IGF2 secretion activates IGF1R signalling in breast cancer cells
As noted, the most up-regulated gene in mCAFs compared to pCAFs was IGF2. Using an ELISA assay, we confirmed that the transcriptional data were correlated with protein levels: IGF2 levels were significantly higher in the CM of mCAFs compared to pCAFs or NFs (Fig. 4D). Interestingly, secreted IGF2 levels increased markedly when there was a physical interaction between mCAFs and MDA-MB-436 cells, an increase that was not observed in direct co-culture with the normal breast MCF10A cells (Fig. 4D) nor when the MDA-MB-436 cells were incubated with the CM from the mCAFs without direct contact between the cells (Supplementary Fig. S6). We then determined that CM from mCAFs increased phospho-Igf1R, p-Stat3, p-Akt and p-Erk levels in MDA-MB-436 cells to levels comparable if not greater than that observed with direct IGF2 stimulation (Fig. 4E), confirming that CM from mCAFs can trigger signalling downstream of the Igf-1R. We found that phospho-Igf1R and phospho-Akt levels in MDA-MB_436 cells were increased to a greater extent by CM from mCAFs compared to CM from pCAFs and NFs (Fig. 4F). Similar results were observed with CM incubated with other triple negative breast cancer cells (BT-20 and MDA-MB-231 cells) (Supplementary Fig. S7A and S7B). These findings suggest that mCAFs more potently induce IGF signalling, consistent with their higher level of IGF2 secretion. Finally, high levels of IGF2 protein were observed in the stroma and the tumor cells of the clinical metastatic lesions from which the mCAFs were derived (Fig. 4G). Furthermore, IGF2 protein expression was also very high in breast cancer metastatic lesions in that developed when MDA-MB-436 breast cancer cells were mixed in a 1:1 ratio with mCAFs (1:1) in mouse xenografts (Supplementary Fig. S3D-S3F).
To further investigate the role of IGF2 signaling in the effects of mCAFs on breast cancer cells, we silenced IGF-1R in MDA-MB-436 cells using siRNA (Supplementary Fig. S8). IGF-1R silencing in MDA-MB-436 cells significantly decreased breast cancer cell migration and invasion compared to a scrambled siRNA control (Fig. 5A). Silencing IGF-1R in MDA-MB-436 and in BT-20 cells markedly decreased clonogenic growth stimulated by CM from the different mCAFs compared to scrambled siRNA controls (Fig. 5B). Taken together, these findings support a role for IGF2 signaling in mediating mCAF effects on breast cancer cell lines.

To further explore the role of IGF2 in mediating the effects of CAFs on breast cancer cells, we used an antibody that binds IGF1 and IGF2, BI836845 or xentuzumab (16), presently under investigation in clinical trials in breast cancer (clinicaltrials.gov NCT03659136). MDA-MB-436 cells were stimulated with IGF2 with or without pre-incubation with BI836845 (100 μg/ml) for 4 hours or grown in CM from the different mCAFs that had been treated or not with BI836845 (100 μg/ml) for 8 hours. We found that BI836845 significantly decreased both IGF2- or CM-induced IGF1R signaling in MDA-MB-436 cells (Fig. 5C). Furthermore, BI836845 significantly reduced CAF-CM-stimulated clonogenic growth of MDA-MB-436 cells (Fig. 5D). Consistent with these in vitro results, we observed that the growth of mouse xenografts of MDA-MB-436 cells co-injected with the BM113 mCAFs was inhibited by BI836845 treatment. On the other hand, BI836845 treatment had no significant effect on the growth of xenografts of MDA-MB-436 cells co-injected with pCAFs (Fig. 5E-5H). Examination of tumor nodules showed markedly reduced levels of phospho-IGF1R in BI836845 treated MDA-MB-436 cells mixed with mCAFs tumors compared with those treated with vehicle (Fig. 5I).

mCAFs have immunosuppressive effects mediated by IGF2 secretion

There is early evidence that IGF2 may have immunosuppressive effects on T cells, by enhancing regulatory T cell functions (12). There is also clear evidence from different model systems that CAFs regulate both the innate and adaptive immune response to tumor through the secretion of multiple cytokines, proteases and growth factors (13, 14). Most of this evidence supports an immunosuppressive effect on T cells and dendritic cells (15). However, all of these studies were performed on pCAFs and not on mCAFs. Compared to pCAFs, our mCAFs showed a marked increase in RNA levels of several genes involved in the immune response such as CCL2 (>3-fold) and CXCL12 (>4-fold, confirmed by ELISA, Fig. 6A) as well as interferon-related genes (Fig. 4B and C). Given these results, we assessed the effects of pCAFs and mCAFs on T cell proliferation. T cells labeled with CFSE from three healthy donors were co-cultured with CAFs for 3 days and stimulated with PMA and ionomycin. We found that co-culture with CAFs significantly decreased the proliferation rate of CD4⁺ T lymphocytes, and that this decrease was greatest in direct co-culture with the mCAF compared with the NF and the pCAF (Fig. 6B-6D). Incubation of T cells with IGF2 by itself also decreased T cell proliferation (Fig. 6B). We also examined the role of the CAFs on longer T cell stimulation, PBMC were stimulated with plate bound anti-human CD3 antibody for two days, with different CAFs co-culture or without. We found that mCAFs (BM87 and BM113) could markedly suppress CD3-induced T cells activation, displaying lower CD69 expression, one the earliest antigens expressed on T cells upon activation, compared with PBMCs alone or with NFs (Norm2) or pCAFs (CAF53) in co-culture (Fig. 6G). These results expand and confirm our knowledge that CAFs from metastatic breast cancer have a stronger ability to suppress T cell activation in response to PMA/IONO or anti-CD3 antibody stimulation than pCAFs or normal fibroblasts.
These results suggest that mCAFs may have more potent immunosuppressive effects on T effector cells than pCAFs and NFs. We then found that inhibiting IGF2 activity with BI836845 partially reversed mCAF suppression of T cell activation (Fig. 6E and 6F), suggesting that both direct pro-tumorigenic and immunosuppressive functions up-regulated by mCAFs may involve IGF-2, and can be targeted by BI836845.

**Discussion**

The notion that the tumor microenvironment plays an essential role in breast cancer biology and therapeutic response is well established. The key pro-tumorigenic function of breast CAFs is well supported by multiple studies including our own (17, 18). However, there is a gap in knowledge concerning the role of CAFs from metastatic lesions. One very recent study compared the estrogen-induced miRNA profile from 1 cutaneous metastatic breast tumor to primary breast CAFs (19). Using our breast tumor biobank, we collected and analysed 8 mCAFs from different sites and performed the first study comparing mCAFs with CAFs from primary breast tumors (pCAFs) sites. In this unique set, we found marked differences in gene expression and ability to stimulate neoplastic growth between mCAFs and pCAFs and NFs. mCAFs conferred stronger growth stimulation and drug resistance on breast cancer cells, and expressed higher RNA levels of growth factors such as IGF-2 and PDGFA than other CAFs. We then found that IGF-2 protein levels were very high in mCAF-CM and that co-culture of mCAFs with MDA-MB-436 cells increased IGF-2 levels even more (2-4 fold), together with IGF signaling activation. We also found evidence that the CM from mCAFs has immunosuppressive activity, in part due to IGF2 paracrine secretion. Finally we observed that the use of an IGF1/2 neutralizing antibody, BI836845, blocked the *in vitro* and *in vivo* mCAF effects on cancer cell growth, IGF-1R signaling, tumor growth and immunosuppressive effects. We also observed that an interferon gene signature was present within the transcriptome of mCAFs, confirmed by higher IFN-ß levels in mCAFs. Indeed, we recently reported that the interferon type I response was activated in a subset of pCAFs (5 of 23 pCAFs or 22%), with pro-mitogenic effects (17).

Our findings are consistent with our recently published work, in which we reported that treatment of a TNBC xenograft model (MDA-MB-231) with an IGF-1R inhibitor resulted in a significant inhibition of lung metastases and not primary tumor growth in vivo (20). Singer et al found that IGF2 and not IGF1 mRNA was increased in primary CAFs and that co-culture with breast cancer cell lines markedly increased IGF2 expression (21). Moreover, high IGF2 expression has been found in CAFs from lung metastatic lesions in syngeneic mammary mouse models (22). Our findings are also consistent with the hypothesis that CAFs have immunosuppressive effects on T cells. CAFs secrete many cytokines that have direct effects on adaptive immunity and immunosuppressive effects have been reported in *in vitro* (14), genetically engineered (23) and syngeneic tumor mouse models such as the 4T1 mouse model (24, 25). Furthermore, CAFs from lung cancers with high Tregs in the stroma were able to induce FOXP3 expression in naïve CD4+ T cells (26). pCAFs have been found to contribute to resistance to anti-PD-L1 treatment in a mouse model of colorectal cancer (24). One can speculate that combining BI835845 with anti-PD-L1 drugs may reveal a new potent therapeutic avenue for tumors expressing both IGF2 and PD-L1.

In summary, we have for the first time analysed a set of CAFs from metastatic sites in breast cancer, and have found significant molecular and phenotypic differences between mCAFs and CAFs from primary breast tumors. These differences may contribute to the intrinsic resistance of...

References


Figure Legends

**Figure 1.** mCAFs promote breast cancer cell proliferation *in vitro* and protect from chemotherapy cytotoxicity. **A,** Cell proliferation was measured by the AlamarBlue assay on MDA-MB-436 cells incubated for three days with CM from different fibroblasts (NF, pCAF and mCAFs) or without CM (control). Results are presented as the mean RFU +/- SEM of triplicate measurements. **p < 0.01; ***p < 0.001 (Student’s *t*-test). **B,** Colony formation assay of MDA-MB-436 and BT-20 cells incubated with CM from NFs, pCAFs or mCAFs and their controls (in regular DMEM medium with 2% FBS). Right panels show the quantitative analysis of three independent formation assays performed, error bars correspond to SEM. *p < 0.05; **p < 0.01; ***p < 0.001 (Student’s *t*-test). **C** and **D,** Quantification of the number of MDA-MB-436 cells that migrated (**C**) and invaded (**D**) from the upper chamber towards the lower chamber of invasion trans-wells containing CM from NFs, pCAFs or mCAFs or regular medium (control). Migrated and invaded cells were stained with crystal violet and counted. The bar graphs represent the mean ± SEM. *p < 0.05; **p < 0.01 (Student’s *t*-test). **E,** Fluorescent microscopy images of GFP-labeled MDA-MB-436 cells (500 cells/well) cultured alone (control) or in direct co-culture with NFs (BERK407 and NORM2), pCAFs (CAF35, CAF53, CAF65) and mCAFs (BM69, BM87, BM113) (5 x 10^4 cells/well) mixed. Following co-culture for about 10 days 3D sphere-like structures formed. The right panel shows the quantitative analysis, the average sphere diameter of ten random colonies from three different experiments were measured using ImageJ software. Scale bar =400 μm. Student’s *t* test analysis **p < 0.01; ***p < 0.001. **F,** GFP-MDA-MB-436 cells (10 000 cells) were mixed with NFs, pCAFs and mCAF at a ratio of 1:3 (436:fibroblasts) in 2% FBS DMEM containing matrigel and seeded in a 96-well plate coated with matrigel. After 10 days of 3D growth doxorubicin (10 μM) or regular medium was added. The representative fluorescent microscope images display the aggregates/spheroids formed after10 days of drug treatment. The average spheroid diameter was quantified with ImageJ software. Bar graph represents the mean ± SEM of three replicate experiments. Statistical analysis was performed using Student’s *t* test.

**Figure 2.** mCAFs enhance tumor formation and metastasis of human breast cancer cells *in vivo.* **A,** Photographs of tumors obtained after breast cancer MDA-MB-436 cells (1x10^6) were injected alone or with NFs, pCAFs and mCAFs (ratio 436:fibroblasts = 3:1) into the mammary fat pads of athymic nude mice (n=5 per group). Tumor growth was monitored and tumor volume measured every 3-4 days. Co-injection with mCAFs resulted in the formation of larger tumors. **B,** Growth curve showing average tumor volume ± SD during tumor growth. Student’s *t* test comparing tumors from CAFs relative to NFs. **p < 0.01. **C,** MDA-MB-436 cells (0.7 x 10^6) were injected with the same number of NFs, pCAFs or mCAFs (ratio 1:1) into mammary fat pads of athymic nude mice (n=3, each group). Tumor growth was evaluated every 3-4 days. **p < 0.01 (Student’s *t*-test). **D,** Mice bearing the tumors are shown. **E,** After the last indicated
measurement, tumors were removed and photographed; primary tumors (pCAF and NF) from three mice and metastatic liver tumors from two mice with tumors co-injected with mCAFs are displayed.

**Figure 3.** mCAFs promote an EMT phenotype in breast cancer cells. **A,** Representative flow cytometry plots depicting the expression of CD44 (FITC labeled) and CD24 (AF647 labeled) in MDA-MB-436 cells cultured alone (control) or with CM from NFs (Norm2), pCAFs (CAF35, CAF53) and mCAFs (BM69, BM87). The percentage of CD44+/CD24- cells is indicated in the top left corner. Right panel is a bar graph showing the average ± SEM of percent CD44+/CD24- cells for 3 experiments. **p < 0.01; ***p < 0.001 (Student’s t-test). **B,** Expression of E-cadherin and N-cadherin proteins in sorted GFP-labeled cells measured by Western blot analysis showing relative increase of N-cadherin and decrease of E-cadherin expression in mCAFs. β-actin was used as a loading control. **C** and **D,** mRNA expression of **SNAIL** (C) and **Nanog** (D) measured by real-time RT-PCR in GFP-MDA-MB-436 cells directly co-cultured with NFs, pCAFs and mCAFs. GFP-labeled cells were separated by fluorescent cell sorting from the different co-cultures and grown alone further in CM from the corresponding CAFs for 8 h in order to ensure the absence of CAFs in RNA and protein analysis. The expression (average ± SEM) of each gene relative to the housekeeping gene **Gapdh** from triplicate measurements of two separate experiments is represented. **p < 0.01; ***p < 0.001(Student’s t-test).

**Figure 4.** Canonical pathway analysis of RNAseq data. **A,** PCA of RNA seq data from NFs (blue), pCAFs (green) and mCAFs (red) shows 3 clusters: upper left: NFs and pCAFs; upper right: cluster 1 mCAFs; lower left: cluster 2 mCAFs. **B,** Gene ontology analysis of up regulated genes (>2 fold, p<0.05, > 10 reads in mCAFs) in mCAFs vs pCAFs was performed with DAVID functional analysis. The top 12 biological processes significantly enriched (Bonferroni corrected p<0.05) are depicted. **C,** Interferon-beta (IFN-β) levels was measured by ELISA assay in CM from corresponding fibroblasts. Student’s t-test was used to compare the mean of groups. **p < 0.01. Note that cluster 1 mCAFs express significantly more IFN-β than cluster 2 mCAFs. **D,** Levels of IGF2 secreted in CM by NFs, pCAFs and mCAFs alone and when directly co-cultured with MDA-MB-436 cells or normal MCF10A breast cells for 3 days. IGF2 concentration was measured by ELISA from triplicate measurements of two separate experiments. *p < 0.05; ***p < 0.001, (Student’s t-test). Note that direct co-culture of MDA-MB-436 cells with mCAFs leads to a marked increase in IGF2 secretion in culture media, not observed with MCF-10A co-culture. **E,** Western blot analysis of IGF2 signaling proteins including Igf1R, phospho-Igf1R (p-Igf1R), Stat3, phospho-Stat3 (p-Stat3), Akt, phospho-Akt (p-Akt), Erk and phospho-Erk (p-Erk) in extracts from MDA-MB-436 cells cultured alone (first lane), co-cultured with CM from mCAFs for 3 days (left half) or after direct stimulation with recombinant IGF2 (20ng/ml). β-actin was used as the loading control. **F,** Western blot analysis of the same IGF2 signaling proteins in MDA-MB-436 cells cultured with CM from different fibroblasts. **G,** Expression of IGF2 in 3 patient tumor samples of primary and metastatic lesions (T511, BM47 and BM69L) analyzed by immunohistochemistry (IHC). High expression of IGF2 was observed in metastatic tissues, in both tumor cells and stromal cells. Photos were taken under a light microscope and digital images (10x) analyzed by ImageScope software. Scale = 200μm.

**Figure 5.** Decreased IGF2 expression or inhibition its receptor IGF1R can reverse CM mediated breast cancer cell proliferation, migration and invasion *in vitro* and *in vivo.* **A,** Transient IGF1-R
depletion resulted in decreased breast cancer cell migration (left) and invasion (right). The bar graphs show the number of migrated/invaded cells in five random fields from three separate experiments (mean ± SEM) are shown. *p < 0.05, Student’s t-test. B, Colony growth of control (scrambled) and IGF1R silenced (siRNA-IGF1R) MDA-MB-436 and BT-20 cells co-cultured with CM from mCAFs for 14 days. Representative images of individual wells from one experiment are shown. Right panels show the quantification of the average number of colonies for both cell lines and conditions of three independent experiments performed. IGF1-R depletion resulted in decreased number of colonies. Statistical analysis was performed using Student’s t-test to compare scrambled vs si-IGF1R *p < 0.05; **p < 0.01. C, Western Blot of IGF2 signaling pathway proteins in MDA-MB-436 cells stimulated with IGF2 (20ng/ml) or incubated with CM from mCAFs with or without the IGF1/2 neutralizing antibody BI836845 (100 μg/ml) for 4 h. D, Colony formation assay of MDA-MB-436 cells exposed to mCAFs CM with or without BI836845 (100 μg/ml). Right panels show the quantitative analysis of three independent colony formation assays, error bars correspond to SEM. **p < 0.01 (Student’s t-test). E-H, MDA-MB-436 cells (10^5) were injected with CAF53 (pCAFs) (E and F) or BM113 (mCAFs) (G and H) (ratio 436:CAF = 3:1) into mammary fat pads of athymic nude mice (n=3, each group). BI836835 (100mg/kg) was administered twice a week for 2 weeks to the mice after palpable tumors were formed. Control mice were injected with the same amount and schedule of IgG. Tumor volume was determined with the use of calipers every 3-4 days. The average tumor volume (mean ± SD) and tumor figures were displayed. I, Western blot showing phospho-IGF-1R and total IGF-1R levels in MDA-MB-436 cells mixed with BM-113 mCAF tumors treated with BI836845 or vehicle.

**Figure 6.** m-CAFs inhibited CD4^+ T lymphocyte proliferation from health donor. A, CXCL12 levels was measured by ELISA assay in CM from corresponding fibroblasts. Student’s t-test was used to compare the mean of groups. ** p <0.01. B, CD4^+ T lymphocytes isolated from healthy donor were stained with CFSE and incubated physically with NFs (Norm2), pCAFs (CAF53) and mCAFs (BM113) or recombinant IGF2 (20ng/ml), plus PMA (50ng/ml) /IONOMYCIN (500ng/ml) stimulation. After 3 days, proliferation of T lymphocytes was evaluated by monitoring dilution of CFSE fluorescence on gated CD4^+ T cells. Note that IGF2 partially suppresses T cell proliferation in all cases. Data shown are from one representative health donor. C, Bar graph showing the results of 3 separate experiments from 3 health donors measuring T cell proliferation, when co-cultured with different fibroblasts with or without PMA/Ionomycin (IONO). Student’s t-test was used to compare the groups. * p<0.05. D, Western Blot showing phospho-tyrosine levels in T lymphocytes cultured with different fibroblasts and stimulated with PMA/IONO for 3 days. β-actin is the loading control. E, CD4^+ T lymphocytes isolated from healthy donor were stained with CFSE and incubated physically with NFs (Norm2), pCAFs (CAF53) and mCAFs (BM113) or BI836835 (100 μg /ml), plus PMA (50ng/ml) /IONOMYCIN (500ng/ml) stimulation. After 3 days, proliferation of T lymphocytes was evaluated by monitoring dilution of CFSE fluorescence on gated CD4^+ T cells. Note that BI836845 only affects the T cells when co-cultured with the BM113 mCAF. Data shown are representative of one health donor. F, Bar graph showing the results of 3 separate experiments from 3 health donors measuring T cell proliferation, when co-cultured with different fibroblasts. Student’s t-test was used to compare the groups. * p<0.05. G, Flow cytometry analysis of CD3+ CD69+ surface fluorophore expression upon PBMCs, which were cultured in LEAF anti-human CD3 (10μg/ml) coated or without 96-well plate, and co-cultured with the
different CAFs. After two days of incubation, CD3+ T cells were gated and histograms of CD69 were analyzed representing changes in mean fluorescence intensity by flow cytometry compared to baseline. Results were shown from a single experiment, representative of two independent experiments. ** p<0.01.
Gui et al., Fig. 1.
Gui et al., Fig. 2.

A

B

C

D

E

- MDA-MB-436/BM113 (mCAF)
- MDA-MB-436/CAF53 (pCAF)
- MDA-MB-436/Norm2 (NF)

Tumor volume vs. Days after tumor injection

- MDA-MB-436/CAF53 (pCAF)
- MDA-MB-436/Norm2 (NF)
- MDA-MB-436/BM113 (mCAF)

Tumor volume vs. Days after tumor injection

- MDA-MB-436/CA53 (pCAF)
- MDA-MB-436/Norm2 (NF)
- MDA-MB-436/BM113 (mCAF)

- Mouse 1# (mCAF)
- Mouse 2# (mCAF)

- MDA-MB-436/CAF53 (primary tumor, pCAF)
- MDA-MB-436/Norm2 (primary tumor, NF)
- MDA-MB-436/BM113 (metastatic tumor, mCAF)
 Gui et al.,

(A) Flow cytometric analysis of CD44^+ CD24^− cells in Control, Norm2, CAF35, CAF53, BM69, and BM87 cells.

(B) Western blot analysis of E-Cadherin, N-Cadherin, and Actin in MDA-MB-36 cells treated with NFs, pCAFs, and mCAFs.

(C) Relative mRNA expression of SNAI1 normalized to gapdh in NFs, pCAFs, and mCAFs in MDA-MB-36 cells.

(D) Relative mRNA expression of Nanog normalized to gapdh in NFs, pCAFs, and mCAFs in MDA-MB-36 cells.
Gui et al., Research. on September 17, 2019. © 2019 American Association for Cancer Research. Published OnlineFirst on September 12, 2019; DOI: 10.1158/1078-0432.CCR-19-1268

**Figure A**

- PC1: 36% variance
- PC2: 15% variance

**Figure B**

- Type I interferon signaling pathway
- Response to virus
- Defense response to virus
- Negative regulation of viral genome replication
- Inflammatory response
- Extracellular matrix organization
- Positive regulation of MAPK cascade
- Immune response
- Cell adhesion

**Figure C**

- IFN-β (pg/ml)
- Cluster 1
- Cluster 2

**Figure D**

- IGF-2 (ng/ml)
- MDA-MB-436
- MCF10A

**Figure E**

- Co-culture with mCAFs CM
- IGF-2 stimulation

**Figure F**

- Western blot analysis

**Figure G**

- T511 (IGF2) skin metastasis
- BM47 (IGF2) liver metastasis
- BM69 (IGF2) liver metastasis
Gui et al.,

**A**

Number of migrated cells

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Number of invaded cells

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**B**

Number of colonies

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IGF2 (min)

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MDA-MB-436

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**E**

Tumor volume (mm)

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**F**

Vehicle BI836845

**G**

Tumor volume (mm)

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**H**

Vehicle BI836845

**I**

436+BM113 tumor

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Gui et al.,

**CXCL12 (pg/ml)**

0 2000 4000 6000

Norm2 Berk047 CAF50 CAF53 CAF57 T511 T563 BM69 BM33 BM47 BM69L BM87

Berk047 CAF50 CAF53 CAF57 T511 T563 BM69 BM33 BM47 BM69L BM87

**N-Fs p-CAFs m-CAFs**

**Gui et al., Fig. 6.**

A B C

T cell T cell/Norm2 T cell/CAF53

T cell/BM113

NO PMA/IONO IGF2/PMA/IONO

1.2 23.6 13.6

1.3 14.2 26.6

1.5 17.5 4.7

D

E

F

G

PMA/IONO PMA/IONO PMA/IONO + BI836845

naive

T cell T cell T cell T cell T cell T cell

CFSE counts

T lymphocytes

MV(kda)

p-Tyrosine

Actin

T lymphocytes

0MV(kda)

Comp-FITC-A

CD69

Coated with anti-human CD3

Mean Fluorescence Index

Coated with anti-human CD3

PBMC+BM87 PBMC+BM113 PBMC+CAF53 PBMC+Norm2 PBMC

PBMC