PREDICTING PATIENT-TO-PATIENT VARIABILITY IN PROTEOLYTIC ACTIVITY AND BREAST CANCER PROGRESSION

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PREDICTING PATIENT-TO-PATIENT VARIABILITY IN PROTEOLYTIC ACTIVITY AND BREAST CANCER PROGRESSION

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SUMMARY

About one in eight women in the United States will develop breast cancer over the course of her lifetime. To make matters worse, patient-to-patient variability in disease progression continues to complicate clinical decisions in diagnosis and treatment for breast cancer patients. Early detection of tumors is a key factor influencing patient survival, and advancements in diagnostic and imaging techniques has allowed clinicians to spot smaller sized lesions. There has also been an increase in premature treatments of non-malignant lesions because there is no clear way to predict whether these lesions will become invasive over time. Patient variability due to genetic polymorphisms has been investigated, but studies on variability at the level of cellular activity have been extremely limited. An individuals biochemical milieu of cytokines, growth factors, and other stimuli contain a myriad of cues that pre-condition cells and induce patient variability in response to tumor progression or treatment.

Circulating white blood cells called monocytes respond to these cues and enter tissues to differentiate into monocyte-derived macrophages (MDMs) and osteoclasts that produce cysteine cathepsins, powerful extracellular matrix proteases. Cathepsins have been mechanistically linked to accelerated tumor growth and metastasis. This study aims to elucidate the variability in disease progression among patients by examining the variability of protease production from tissue-remodeling macrophages and osteoclasts. Since most extracellular cues initiate multiple signaling cascades that are interconnected and dynamic, this current study uses a systems biology approach known as cue-signal-response (CSR) paradigm to capture this complexity comprehensively.

The novel and significant finding of this study is that we have identified donor-to-donor variability in disease modifying cysteine cathepsin activities in macrophages and osteoclasts. This is the first study that has explicitly and extensively investigated inter-donor variability in cellular proteolytic activity and predicted the donor-specific cathepsin activity with greater than 90% predictability. This study applied this novel finding to the context of tumor invasion and showed that variability in tumor associated macrophage cathepsin activity and their inhibitor cystatin C level mediates variability in cancer cell invasion. Furthermore, by inhibiting JNK activation of c-Jun, a kinase that has been linked to increase in cathepsin activity, and those individuals whose macrophages had the highest cathepsin activity and the greatest invasive potential saw the greatest reduction in these outcomes. Monocytes from these individuals had low level of JNK activation.

These findings help to provide a minimally invasive way to identify individuals with particularly high remodeling capabilities. This could be used to give insight into the risk for tumor invasion and develop a personalized therapeutic regime to maximize efficacy and chance of disease free survival.

CHAPTER I

INTRODUCTION

About one in eight women in the United States will develop breast cancer over the course of her life time.[142] However, patient-to-patient variability in disease progression continues to complicate clinical decisions in diagnosis and treatment for patients.[179, 152, 27, 40, 153, 180, 205] Although advancement in diagnostic and imaging techniques has led to an increase in early detection of breast cancer, this increase is also accompanied by a downside of premature treatment of non-malignant lesions, because there is no clear way to predict whether these lesions will become invasive over time. Patient variability due to genetic polymorphisms has been investigated [71, 61], but studies on variability at the cellular proteolytic activity level have been extremely limited.[47] An individuals biochemical milieu of cytokines, growth factors, and other stimuli contain a myriad of cues that pre-condition cells and induce patient variability in response to disease progression or treatment. The focus of this work is on investigating this patient variability at the cellular activity level and elucidating the underlying mechanism for the variability in order to develop personalized medicine strategies for tissue remodeling diseases such as cancer and cardiovascular diseases.

1.1 Interpatient variability in breast cancer

A recent study showed that the incidence of breast cancer overdiagnosis may be as high as 30%.[11] Because currently, there is no good method to predict invasive potential of non-malignant lesions, some women choose to undergo radical mastectomy to prevent malignant progression. In fact, the lack of ways to predict patient-specific disease potential and rate of progression is not confined to breast cancer, but extends

to other serious and prevalent illnesses including other cancers and cardiovascular diseases.

Advances in genomic analysis have lead to discovery of many genetic markers that predispose a person to certain diseases with high risk factors, such as the BRCA1 and BRCA2 gene mutation for breast and ovarian cancer. Although 70% percent of women with these mutations do develop cancer, 30% do not. Various environmental and other molecular modifiers affect risk predictions.[136] A number of studies that identified vast intratumoral heterogeneity within one patient further multiplies the complexity of determining prognosis and treatment regimens. [161, 53, 144, 8] It is clear that a one-size-fits-all approach of diagnosis and treatment is not the most effective or cost-efficient way to treat and eliminate multifactorial diseases. There is clearly a need for a personalized approach.

1.2 A need for personalized medicine

Physicians have explored the idea of personalized medicine by taking a trial-and-error approach to find best course of treatments for individual patients.[41] In more systematic ways, *n=1* or single subject clinical trials have been conducted since the since 1986.[63, 64, 98, 79] In these studies, each patient was his or her own control and the outcomes of varying treatment strategies were compared to the control group. Although these approaches showed efficacy, it was time-consuming and patients underwent various trials before finding the best treatment. The ultimate goal of personalized medicine is to avoid this trial-and-error period and to be able to predict disease progression, optimized treatment regimen, and the outcomes *a priori* for each patient. In the field of pharmacology, improved understanding in biochemistry, molecular biology, and pharmacodynamics combined with advancement of computational science has allowed effective rational drug design. Although the laws that

govern human body are complex, new readily available personalized genomic and proteomic information as well as computational and experimental tools to process and integrate a large quantify of data efficiently, will allow us to revisit the idea of *n=1* methodologies to treat patients.

1.3 Roadmap to personalized medicine: considering possibility versus reality

As we discussed briefly earlier, genetic predisposition is just that, a genetic tendency or possibility to develop certain diseases. Since Francis Crick described the idea of the central dogma of molecular biology where genetic information flows from DNA to RNA to protein [29], scientists have found a number of ways that provided exceptions to the rule from epigenetic changes to regulations at the protein level. When determining a persons chance of developing an invasive breast cancer from the initial overgrowth, what needs to be considered is not only their genetic risks but also cellular effectors namely proteases that execute the pathogenesis and disease progression. Unlike genetic variability among individuals, studies on variability in cellular proteolytic activities has been extremely limited.[47]

Cysteine cathepsin proteases are potent collagenases and elastases whose expression and activities are tightly regulated at multiple points along the central dogma. Of particular interest to this current study occurs after translation. Like many other protease families, cathepsins are synthesized as inactive precursors (procathepsins) and to be active, cleavage of a propeptide occurs by other proteases or through autocatalytic mechanisms.[23, 129, 132, 196] Once active, cathepsin proteolytic activity is regulated by environmental factors such as temperature, pH, and oxidative potential. Cathepsins are optimally active at acidic pH, and prefer reducing environments for the SH group of the active site cysteine to participate in the nucleophilic attack that cleaves peptide bonds.[149, 33] Finally, the cystatins are a family of cysteine cathepsin inhibitors that regulate and inhibit intra- and extracellular cathepsin

activity.[128, 190, 78, 192, 194] Taken together, *in vivo*, cathepsins exist as a system of relatively short-lived enzymes working simultaneously on multiple substrates, both intra- and extra-cellularly.[118] We have shown that there are appreciable patient-to-patient variability in the amount of active cysteine cathepsin protease in monocyte-derived macrophages and osteoclasts[155] and in cystatin C level of macrophages. As it will be discuss further, cysteine cathepsins play central mechanistic roles in cancer and cardiovascular diseases. These findings emphasize the need to investigate patient variability at the level of active proteases for personalized diagnosis and treatments.

1.4 Macrophages, osteoclasts and cathepsins in cancer

Monocytes are circulating white blood cells that respond to milieu of cytokines, growth factors, and other stimuli, and enter tissues to differentiate into monocyte-derived macrophages (MDMs) and osteoclasts. They actively participate in tissue remodeling by producing cysteine cathepsins. Cathepsins produced by macrophages and osteoclasts have been mechanistically linked to accelerated tumor growth and metastasis.[56, 138, 197, 112, 167] The variability in patients cathepsin protease production by MDMs and osteoclasts due to the varying biochemical stimuli can be employed to elucidate the variability in disease progression that involves cathepsins.

Cysteine cathepsins are included in the papain family of proteases that comprises 11 members denoted by letters: cathepsins B, C, F, H, K, L, V, O, S, W, and Z (or X). In humans, cathepsins were first identified in lysosomes for their role in protein turnover, but are now known to play functional roles in other cellular compartments and even in the extracellular space after secretion.[16, 23, 38, 194] Cathepsins are optimally active in slightly acidic and reducing environments. This is one benefit for personalized medicine in that they are relegated to specific cell types and subcellular compartments under healthy conditions, but then are turned on in different cells and cellular subcompartments during disease, lending them to

being useful biomarkers.[177, 163, 60] As an example, cathepsin K is the most potent human collagenase[51] and was identified in osteoclasts, where it is key for bone resorption. Cathepsin K is now known to be expressed in multiple cell types including macrophages, fibroblasts, dendritic cells, chondrocytes, endothelial cells, smooth muscle cells, and transformed epithelial cells in diseased conditions.[19, 188, 69, 20, 162]

Other cathepsins of interest for personalized medicine include L, S, and V, which are in the same subclade as cathepsin K. They too have limited cell-specific expression under healthy conditions but get turned on in disease conditions in other cell types.[163, 208] Along with cathepsin K, they can be secreted under disease conditions as well and have been implicated in extracellular matrix degradation in pathological disease progression.[42] Cathepsins K, S, and V are all strong elastases, with cathepsin L having mild collagenase activity.[208, 42, 108]

Until recently, many studies have focused on identifying characteristics of cancer cells themselves. However, it has become clear that tumor microenvironment, which encompasses tumor cells, stromal cells, and recruited immune cells, which include tumor associated macrophages (TAMs), play crucial roles in determining behavior of cancer cells.[111, 43, 49, 28] TAMs that are differentiated from circulating monocytes promote angiogenesis [102, 103, 104, 37, 165, 131], tumor growth[103], invasion, and metastasis[109] through secretion of cytokines to coordinate tumor-promoting immune responses[6, 175, 120, 123] as well as through secretion of tissue-remodeling cathepsin proteases.[80, 55]

In tumor tissues, cathepsins are highly upregulated in both cancer cells and TAMs. Although cathepsins were first discovered in lysosomes, they are now known to be secreted by different cell types including macrophages and osteoclasts.[206, 50] In cancer cells, there is increase in both intracellular and secreted cathepsin activity and this increase is regulated at the transcriptional, translational, and post-translational level. *In vivo* studies on breast cancer have shown that cathepsins L and K promote

invasion [90], and cathepsin B promotes tumor growth, invasion and metastasis to bone.[203] Recently, it was shown that loss of BRCA1 tumor-suppressor gene activates cathepsin L-mediated degradation of 53BP1, which then allows cells to bypass cellular check points and promotes tumor growth.[62] Cathepsins K and L are shown to be elevated in ductal carcinoma *in situ* (DCIS).[4] In addition, it has been shown that cathepsin secretion by cancer cells can be induced by interaction with extracellular matrix (ECM) protein such as collagen I.[160]

Cathepsins secreted by infiltrating tumor-associated macrophages have also been shown to promote cancer cell invasion[197] and to blunt effectiveness of chemotherapy against breast cancer cells.[172] For prostate cancer, cathepsin B was shown to promote angiogenesis and invasion[143] and cathepsin S secreted by infiltrating macrophages were associated with poorly differentiated tumors and castration-resistant tumors.[110] For pancreatic cancer, cathepsins B and S have been shown to promote angiogenesis and tumor formation[57] and cathepsins B and L promote cancer cell proliferation and growth.[57, 200] Lack of cystatin C, an endogenous inhibitor of cysteine cathepsins, increased the number of pre-malignant lesions. On the other hand, tumor metastasis to lung but not to liver, two of common metastatic sites, was reduced when cystatin C was overexpressed. Taken together, specialized role of individual cathepsins in multiple stages of tumor progression and by different cell types provide opportunities for tailored prognosis as well as targeted therapies to minimize undesired effects.

The role of cathepsin K, traditionally known to be secreted by osteoclasts, have been investigated mostly in the context of metastasis to bone, which is a common metastatic site for prostate, breast, and lung cancer. These metastatic cancer cells have shown various osteoclast-stimulating properties. [25, 189, 181] Increase in osteoclast differentiation or stimulation of osteoclasts leads to increase in cathepsin K secretion and bone resorption, which in turn releases growth factors from bone matrix

and stimulates cancer cells.[87] Consequently, cathepsin K inhibitors have been suggested for treatment of osteolytic bone metastasis[99], but no data from large-scale clinical trial is currently available. A number of cathepsin K inhibitors have been developed to treat osteoporosis, but only one of them, odanacatib, has completed phase III clinical trial.[18] Cathepsins have been shown to promote cancer cell migration and invasion [92, 139, 93], potentially through cleavage of extracellular proteins[119, 72] and cell adhesion proteins as well as intracellular degradation of matrix proteins such as collagen.[57] Secreted cathepsins have been linked to neoplastic transformation and intracellular cathepsins have been linked to tumor invasion.

1.5 Kinase signaling network in understanding patient-specific cell behavior

Despite crucial roles monocyte-derived macrophages and osteoclasts have in diseases, how monocytes interpret environmental cues, process signals, and respond are not well understood. Intracellular signal propagation is often transient, and the traditionally examined cell surface markers and clusters of differentiation (CD) may not reflect this within the critical time frame and requires novel descriptors of cell state. Moreover, because most extracellular cues initiate multiple signaling cascades that are interconnected and dynamic, a system-wide approach must be taken to accurately capture this complexity. One tool that has been used successfully to extract important biological information by integrating unknown and measurable variables at a nexus, is the cue-signal-response paradigm (Figure 2). The cue-signal-response paradigm integrates multiple extracellular cues received by cells, measures changes in the induced signals, and interprets cell decisions to execute responses (Figure 2).[75, 159, 74, 134] In particular, computational analysis of dynamic changes in kinase activation has shown that kinases serve as integrators of stimuli from different soluble, cellular, and physical cues, to generate specific cellular responses.[77, 201, 101, 183] Prediction of monocyte

differentiation and resultant proteolytic activity from an individual signaling pathway in isolation is problematic due to complex crosstalk in signaling networks, but is substantially improved when multiple pathways are considered (Figure 2).[135, 75]

1.6 Partial least square regression (PLSR)

PLSR is based on principal component analysis (PCA), where based on principles of eigenvectors and eigenvalues, data sets are organized into a MxN data matrix X (Figure 2) and new variables, called principal components (PCs), are calculated that capture most of the information contained in the data set based on covariance between measurements. This approach de-emphasizes measurements that are noise and shows little covariance with other measurements and highlights the measurements that covary together and identifies a global pattern in the data set. For PLSR analysis, covariance of independent variables in the X-matrix is calculated based on how well they describe or predict the dependent variables in the Y-matrix. Variable importance in projection (VIP) scores can be calculated to identify independent variables that highly contribute or are predictive of the dependent variable. For each independent variable, regression coefficient is also calculated where $Y = XB + E$ and B is regression coefficient matrix and E is residual noise. This coefficient matrix $\mathbf{B}$ is referred to as a PLSR model and can be used to predict responses for a whole new set of independent measurements (Figure 3).[76, 3]

1.6.1 Use of PLSR in biology

Partial least square regression (PLSR) analysis have been used in biology to generate new hypotheses that can be tested experimentally to improve our understanding of biology.[75] One of the advantages of PLSR analysis is that it can find new unbiased relationships within large number of measurements based on global variations and covariance. In systems biology, Cue-Signal-Response (CSR) paradigm refers to cellular

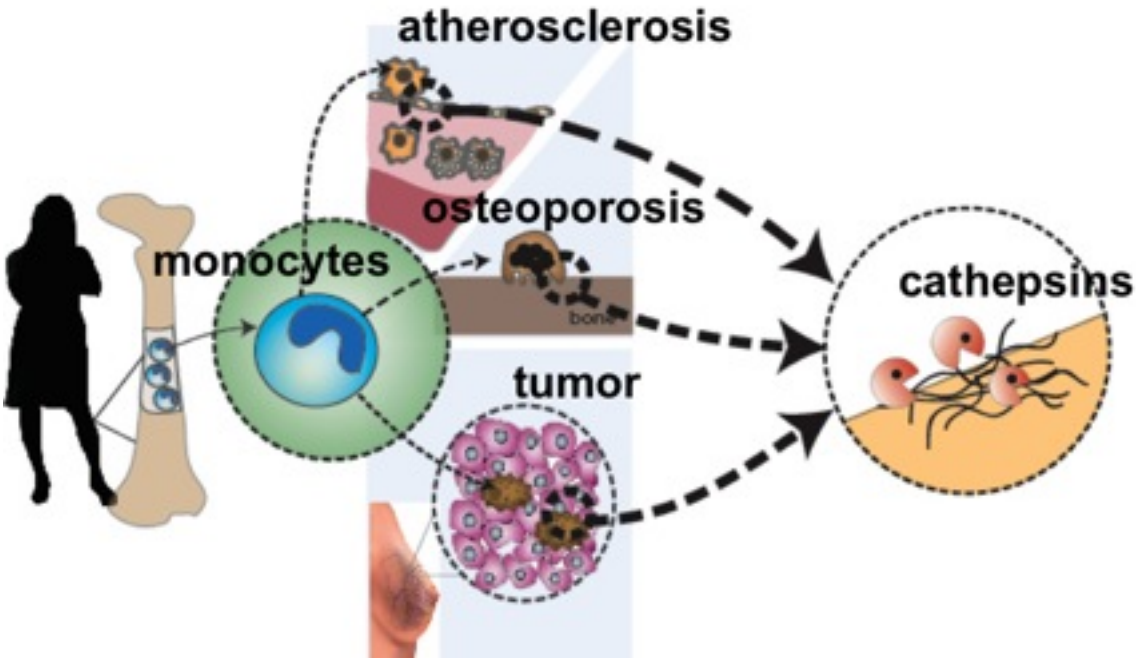

Figure 1: Cathepsins in diseases A schematic showing the role of monocyte derived macrophages and osteoclasts, as well as cathepsins secreted by these cells in atherosclerosis, osteoporosis and tumor.

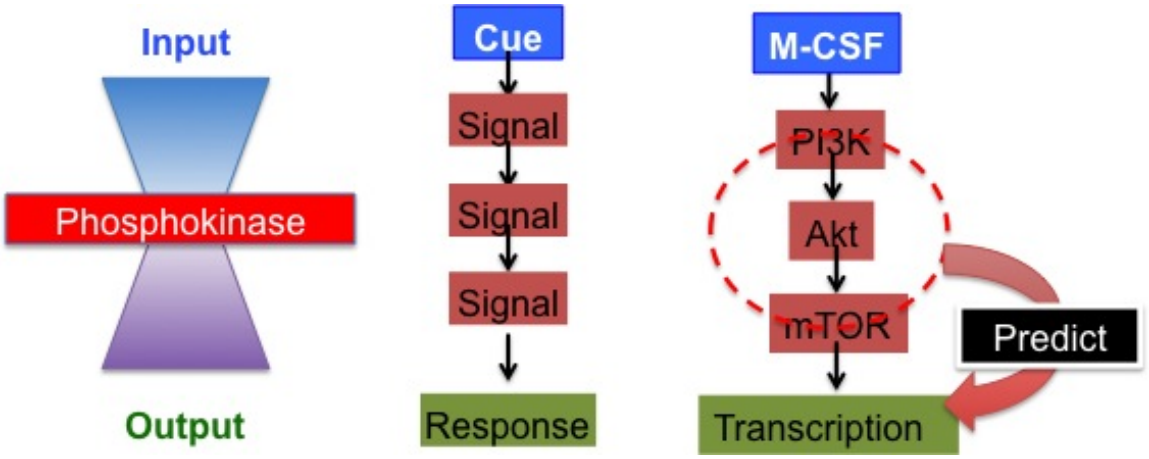

Figure 2: A schematic of cue-signal-response (CSR) paradigm Kinases are signal integrators. When a cell receives some input (cue) from its environment, signals are propagated through activation / deactivation of multiple kinases and result in changes in cellular behavior or responses (gene apoptosis, differentiation, proliferation etc.).

systems where extracellular cues such as cytokines and growth factors activates intracellular signaling network which leads to functional responses by cells. For example, M-CSF (cue) binds to its receptor and the information is propagated by activation of multiple kinases (signal) leading to transcription of genes encoding cathepsins K, S, L, and V.[155] A PLSR model can be generated where the kinase measurements populate an independent matrix X and cathepsin activity measurements populate a dependent matrix Y. Cellular proteolytic activity when stimulated with IL-4 can then be predicted based on new set of kinase signals measured. Also, kinase signals that co-vary with responses could have biological connections that maybe known or to be tested experimentally.

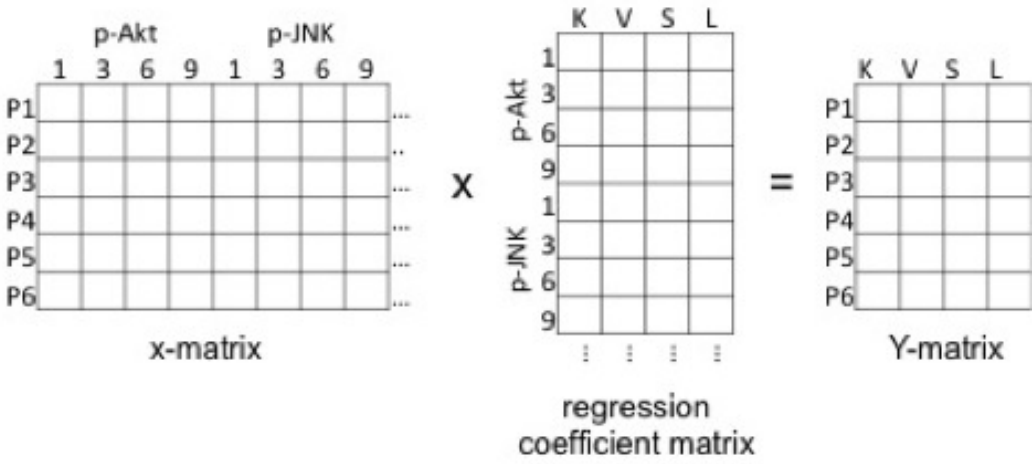

Figure 3: PLSR data matrix X and Y data is generated using signals (X) and responses (Y). Using PLSR analysis, regression coefficient matrix is computed and can be used to predict responses based on new signal matrix.

CHAPTER II

SPECIFIC AIMS AND HYPOTHESES

The central hypothesis of this work is that by interpreting the kinase signaling networks of differentiating monocytes, we can understand patient-specific differences in monocyte differentiation and predict cathepsin-mediated tissue remodeling for individual patients.

2.1 Aim 1. Predict patient-specific cathepsin activity of monocyte-derived macrophages and osteoclasts using multivariate analysis of phosphokinases

Hypothesis: Multivariate analysis of phosphokinase will predict patient-specific morphology of differentiated macrophage and osteoclast and their cathepsin activity. Freshly isolated peripheral blood mononuclear cells (PBMCs) from donors were differentiated into macrophages or osteoclasts and cultured for 14 days. On days 1, 3, 6 and 9, cell lysates were collected for Bioplex kinase analysis of 7 kinases implicated in monocyte differentiation. Mean-centered kinase signals as well as cathepsin activity measured with multiplex cathepsin zymography were input into data-matrices for PLSR analysis. On day 15, cell lysates and conditioned media were collected for cathepsin zymography and western blot to characterize activity and presence of intracellular and secreted cysteine cathepsins K, L, S and V. Cathepsin activity was quantified by densitometry.

2.2 Aim 2: Identify mechanisms for patient variability in cathepsin-mediated disease outcomes.

Hypothesis: Higher macrophage cathepsin activity is associated with increased severity or faster progression of diseases. Freshly isolated PBMCs were differentiated into

macrophages and kinase signals, cathepsin activity and cystatin C level was measured. In vitro co-culture system was used to test variability in cancer cell invasion as proxies for disease progression to test the hypothesis that patent-specific variability in cathepsin activity results in patient-specific variability in disease progression.

The purpose of this work was to identify patient-variability in cellular proteolytic activity as well as to elucidate the underlying mechanism and its implication on the development and progression of tissue remodeling diseases to achieve the ultimate goal of personalized and predictive medicine.

CHAPTER III

DONOR SPECIFIC PROTEOLYTIC ACTIVITY OF MONOCYTE-DERIVED MACROPHAGES AND OSTEOCLASTS PREDICTED WITH TEMPORAL KINASE ACTIVATION STATES DURING DIFFERENTIATION

3.1 Introduction

Patient-to-patient variability in disease progression continues to complicate clinical decisions of treatment regimens for both cardiovascular diseases and cancer that originates at different sites and is diagnosed at different stages of progression. Monocytes are circulating white blood cells that participate in pathogenesis of cardiovascular disease and cancer in response to cues from cells or the environment by leaving the vasculature, entering the tissue, and differentiating into macrophages or osteoclasts. In atherosclerotic lesions, monocyte derived macrophages ingest lipids, become foam cells, and contribute to plaque growth and extracellular matrix (ECM) degradation and remodeling.[169] In cancer, monocyte-derived tumor associated macrophages (TAMs) contribute to almost 50% of the tumor volume and promote tumor invasion, migration and metastasis.[56, 57] Osteoclasts are the multinucleated cells that resorb bone by secreting high levels of cathepsin K to cleave type I collagen, the major structural protein in bone.[171, 17] Osteoclasts have been implicated in later stages of atherosclerotic plaque calcification and shown to be differentiated from infiltrated monocytes.[2, 96, 133] In cancer, osteoclasts are involved in positive feedback loops to develop osteolytic bone lesions with metastasized cancer cells.[99, 141]

Once differentiated from monocytes, both macrophages and osteoclasts contribute to tissue remodeling through the production and secretion of cysteine cathepsins, proteases that have been identified as the most potent mammalian collagenases and elastases that, upon secretion, locally degrade collagen, elastin, and other ECM substrates.[208, 51] Cathepsins K, L, S, and V produced by macrophages and osteoclasts are highly implicated in atherosclerotic vascular remodeling as well as tumor associated tissue remodeling.[115, 116, 157, 158, 184]

Pathologically overactive osteoclasts generate elevated levels of cathepsin K and are the main etiological agents of osteoporosis and osteolytic lesions. Despite the number of pharmacological inhibitors being developed to block this activity, many are failing clinical trials.[99, 18, 36] This may be due to variability in cathepsin activity among patients, altering pharmacokinetics, and in turn, increasing side effects due to under- or over-dosing. Studies have measured circulating cathepsin levels in patients with similar diseases and shown a wide range of variability, whether measured in plasma or serum, among healthy or diseased patients.[113, 96, 94, 84, 2, 75, 159, 74, 134] In particular, computational analysis of dynamic changes in kinase activation has shown that kinases serve as integrators of stimuli from different soluble, cellular, and physical cues, to generate specific cellular responses.[77, 201, 101, 183] Prediction of monocyte differentiation and resultant proteolytic activity from an individual signaling pathway in isolation is problematic due to complex crosstalk in signaling networks, but is substantially improved when multiple pathways are considered.[134, 75] A study used activation of just seven kinases in adult bone marrow-derived stem cells to show that osteogenic differentiation decisions were encoded in temporal kinase activation profiles, and analyzed them with the multivariate analytical technique partial least squares regression (PLSR) to predict terminal differentiation outcomes and phenotype.[159]

In the current study, we investigated if monocyte differentiation into macrophages

or osteoclasts, and the resulting cathepsin activity of these cells were encoded in kinase activation profiles, and if data-driven, multivariate analysis models could predict donor-specific cell differentiation and proteolytic activity. We hypothesized that the temporal kinase activation states would be predictive even with the challenge of incorporating complex unknown cues provided by the genetic and biochemical background of each individual donor that leads to variability.

3.2 Methods

3.2.1 THP-1 Cell Culture

Human THP-1 acute monocytic leukemia cells (American Type Culture Collection [ATCC]) were cultured in RPMI medium 1640 (Mediatech) containing 10% fetal bovine serum (FBS, Atlanta Biologicals), 0.05% -mercaptoethanol, 1% L-glutamine, and 1% penicillin/streptomycin (Life Technologies). Cells were maintained with 5% CO_2 at 37 °C. For macrophage differentiation, monocytes were incubated with 100 nM phorbol myristate acetate (PMA, Sigma-Aldrich) for 24 h, followed by incubation for an additional 11 days in growth medium, with media changed twice per week. For osteoclast differentiation, monocytes were incubated with 100 nM 1,25-dihydroxyvitamin D_3 (Alfa Aesar) for 12 days, with medium changed twice per week. For all differentiation, cells were seeded at 300,000 cells/cm^2.

3.2.2 Primary monocyte isolation and differentiation

Heparinized venous blood from healthy volunteers was diluted 1:1 in sterile PBS, layered on Ficoll-Paque (GE healthcare), and centrifuged at 400g for 30 minutes. The buffy coat layer was isolated, red blood cells lysed, and peripheral blood mononuclear cells (PBMCs) were washed 3 times in PBS. Monocytes adhered overnight and all other non-adherent cells were removed. For macrophage differentiation, isolated monocytes were cultured in RPMI containing 10% male human serum and 30ng/μl

macrophage colony stimulating factor (M-CSF, Peprotech). For osteoclast differentiation, isolated monocytes were cultured in alpha-MEM (Life Technologies) supplemented with 10% fetal bovine serum, 30ng/μl M-CSF, and 30ng/μl receptor activator of NFκB Ligand (RANKL) (Peprotech). Medium was replaced every 3 days.

3.2.3 TRAP Histological Staining

On day 15, both macrophages and osteoclasts were stained for tartrate-resistant acid phosphatase (TRAP) activity according to the manufacturers instructions (Sigma-Aldrich). TRAP activity was visualized under a light microscope as brown and dark red areas. Multinucleated cells were deemed as those with three or more nuclei.

3.2.4 Flow Cytometry

TRAP activity was quantified using flow cytometry modified from an existing protocol and using Fast Red violet (Sigma Aldrich) instead of Fast Garnet GBC. Adherent cells were released using 2 mM ethylene diamine tetraacetate (EDTA), fixed with 4% paraformaldehyde and permeabilized using 0.02% Triton X-100. Cells were then incubated in 2X TRAP Staining Solution (8% of 12.5 mg/ml napthol-ASBI phosphate, 2% of 10 mg/ml Fast Red violet diluted in solution containing 50 mM MES, 50 mM Na Tartrate and pH at 6.3) at room temperature for 9 minutes. Reaction was ended using ice cold PBS. TRAP activity was detected at 488 nm excitation, 610 nm short pass dichroic and measured through a 675$\pm$20 narrow bandpass filter. For CD68 labeling, adherent cells were released using 2 mM ethylene diamine tetraacetate (EDTA), fixed with 4% paraformaldehyde. Cells were incubated in mouse anti-CD68 antibody (Millipore) at 4 °C for 30 minutes. They were then incubated with donkey anti-mouse Alexafluor 488 (Life Technologies) at 4 °C for 30 minutes. Data were expressed as percent of the total cell population positive for CD68.

3.2.5 Cell morphology measurements

Cell diameter and number of nuclei measurements were used for cell morphology. For macrophages, 20 cells were measured per donor, and a single diameter was measured for each cell. For osteoclasts, the number of cells measured varied from 5 to 20 as only cells with more than 3 nuclei were included; due to their irregular shapes, the narrowest and widest parts of each cell were measured and averaged to approximate diameter.

3.2.6 Kinase phosphorylation analysis with Bioplex assays

Differentiating cells were lysed and total protein concentration was determined using microBCA assay (Pierce). Bioplex®bead kits (BioRad) were used according to manufacturers instructions with 5 g protein from each sample. Phosphorylation of ERK1/2 (Thr^{202}/Tyr^{204}, Thr^{185}/Tyr^{187}), Akt (Ser^{473}), p38 MAPK (Thr^{180}/Tyr^{182}), JNK (Thr^{183}/Tyr^{185}), c-jun (Ser^{63}), NFκB p65 (Ser^{536}) and IκB-α (Ser^{32}/Ser^{36}) were measured. Signal values for each phosphorylated kinase were normalized to the signal detected in a master lysate prepared in bulk from pre-stimulated monocytes that was used as a control for all assays. Signal values for each kinase were normalized between 0 and 1 by dividing by the maximum value over the entire 9 days for all treatments.

3.2.7 Partial least square regression (PLSR) analysis

$M\ x\ N$ data matrix was generated with data from M donors and N kinase phosphorylation signals. Each column of the independent X matrix corresponds to a unique input or signal: phosphorylated kinase signal from days 1, 3, 6, and 9, and each column of the dependent Y matrix corresponds to unique outputs. Each row represents a unique donor and stimulation condition (i.e. donor 1- MCSF, donor 1-RANKL, donor 2- MCSF, etc.). All data was mean-centered and scaled to unit variance. SIMCA-P (UMetrics) was used to solve the PLSR problem with the nonlinear iterative partial least squares (NIPALS) algorithm.[52]

3.2.8 Multiplex cathepsin zymography

Media was replaced with serum-free media on day 14 and incubated overnight. This conditioned media was collected and concentrated using VivaSpin500 Centrifugal Concentrator (Vivaproducts). Cellular protein was extracted in lysis buffer (20 nM Tris-HCl at pH 7.5, 5 mM EGTA, 150 mM NaCl, 20 mM -glycerol-phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.1% Tween-20) with 0.1 mM leupeptin freshly added. Cathepsin zymography was performed on cell extracts and on conditioned media as described previously.[202] Briefly, equal amounts of protein in non-reducing loading buffer were separated on 12.5% SDS-polyacrylamide gels containing 0.2% gelatin at 4 °C. Enzymes were renatured and then the gels were incubated overnight at 37 °C in acetate buffer, pH 4 with 1 mM EDTA and freshly added 2mM dithiothreitol. Gels were then rinsed, stained with Coomassie blue, and imaged using an ImageQuant LAS 4000 (GE Healthcare). Densitometry was performed using ImageJ to quantify the intensity of the white cleared band of proteolytic activity. For cathepsin K inhibitor studies, gels were incubated overnight at 37 °C in the presence of 1M cathepsin K inhibitor (1-(N-benzyloxycarbonyl-leucyl)-5-(N-Boc-phenylalanyl-leucyl) carbohydrazide [Z-L-NHNHCONHNH-LF-Boc], EMD Biosciences) or vehicle. [94]

3.3 Results

3.3.1 THP-1 monocyte differentiation into macrophages or osteoclasts is encoded in temporal kinase activation states

Clonal THP-1 monocyte cells were used to identify a set of kinases for predicting macrophage or osteoclast differentiation outcomes. These cells have been differentiated into macrophages with phorbol 12-myristate 13-acetate (PMA) and into osteoclasts with 1,25α vitamin D_3 (1,25 D_3).[170, 70, 146, 89] We first confirmed this in our hands by stimulating THP-1 cells with 100 nM PMA for 1 day, followed by 8

days of culture, or 100 nM 1,25α vitamin D_3 for 12 days. After 12 days, osteoclast differentiation was confirmed by tartrate resistant acid phosphatase (TRAP) positive staining and multi-nucleation (n$\geq$3) as indicated by Hoechst staining (Figure 4A).

In parallel, lysates were collected on days 1, 4, 6, and 9 of differentiation for Bioplex phosphorylated kinase assays. Six μg of total protein was used to quantify phosphorylation of Akt, ERK1/2, p38, IκB, and JNK at each of these time-points in cultures stimulated with either PMA or 1,25 D_3. These kinases were chosen as they have all been implicated during monocyte differentiation into either macrophages, osteoclasts, or both.[170, 168, 146, 15, 187] Kinase phosphorylation signals were normalized to the maximum activation across all conditions for all time points and treatment conditions (Figure 4B). The plots illustrate the difficulty in identifying patterns to link the cues, signals, and responses of differentiating cells based on an individual pathway.

Quantitative measurements of macrophage and osteoclast differentiation were necessary to populate a response matrix for the partial least squares regression (PLSR) model, and establish a mathematical relationship between kinase phosphorylation signatures and monocyte differentiation responses. To quantify macrophage differentiation, flow cytometry was performed after labeling the cells with an anti-CD68 antibody. For osteoclasts, traditional, qualitative colorimetric TRAP staining was modified with a fluorogenic phosphatase substrate to enable fluorescent TRAP activity quantification by flow cytometry.[44] As expected, there was a significantly greater number of TRAP+ cells after stimulation with 1,25 D_3 compared to the other two conditions, while treatment with PMA significantly increased the percentage of cells positive for CD68 expression (Figure 4C, n=3, p¡.01).

With quantitative signals from kinase phosphorylation measurements (Figure 4B) and quantified differentiation responses from flow cytometry (Figure 4C), we were then able to determine if these kinase activation states could be used to predict

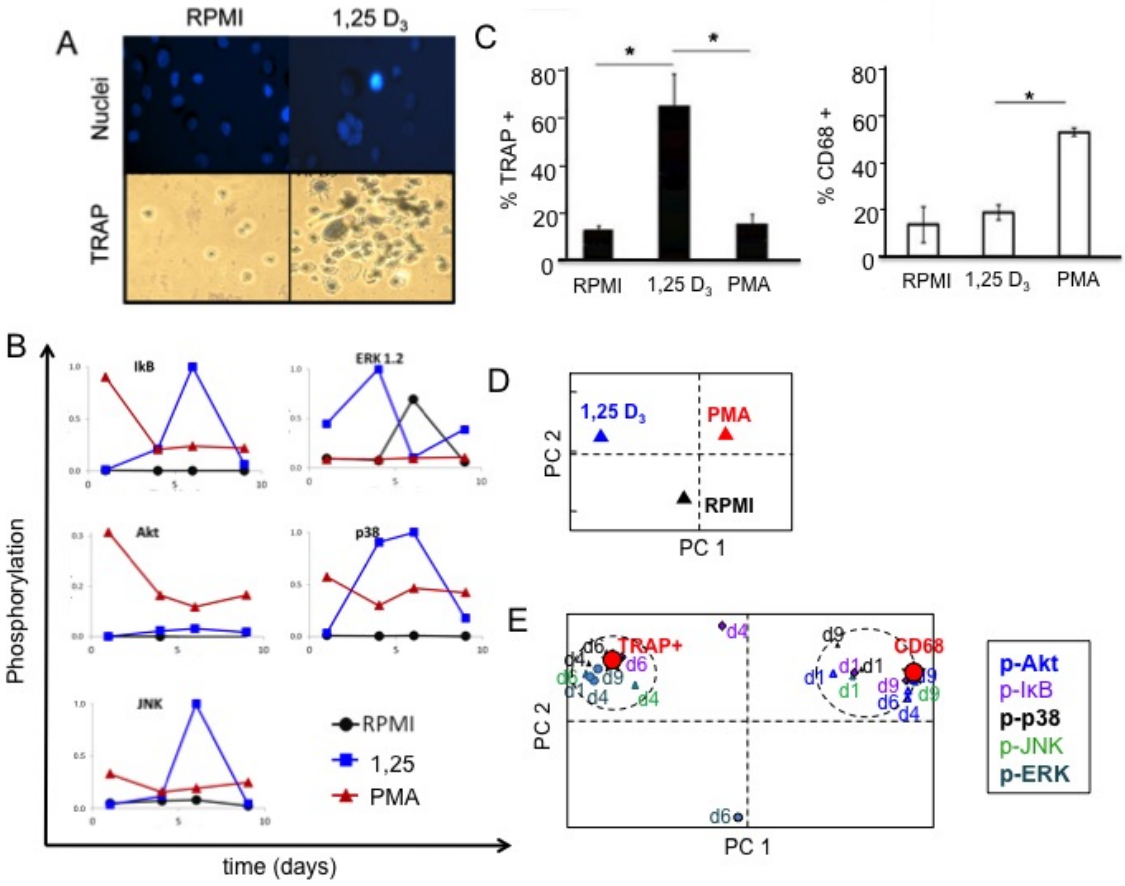

Figure 4: Multivariate analysis of kinase signals of THP-1 monocytes predicts and distinguishes macrophage and osteoclast differentiation outcomes. THP-1 monocytes were stimulated with 100nM PMA or 100nM 1,25 vitamin D_3 (1,25 D_3) for 12 days. A) Nuclei were stained with Hoechst for determining multinucleation (top row). Cells were fixed and stained with colorimetric TRAP activity assay (bottom row). B) On days 1, 4, 7 and 9 of differentiation, macrophages and osteoclasts were lysed and kinase signals were quantified using Bioplex technology and normalized to maximum signal over the time period. C) On day 12, flow cytometry for TRAP activity and CD68 expression was performed. D) PLSR analysis was performed using kinase signals and the scores plot shows polar separation of osteoclasts (1,25 D_3) and macrophages (PMA) along principle component 1 (PC1). E) The loadings plot shows polar separation of temporal kinase phosphorylation and covariance with differentiation outcomes of osteoclasts (TRAP+) or macrophages (CD68).

macrophage or osteoclast differentiation responses. Based on the principles of eigenvectors and orthogonal transformation, PLSR algorithm groups signals with co-varying responses, and reduces dimensionality of the data by plotting them along principal component (PC) axes that capture maximal variance. The PLSR algorithm then computes a linear solution in principal component space based on the proposed relationship between the independent variables X (signals) and dependent variables Y (responses) to calculate a coefficient matrix such that $Y=F(X)$ in principal component space. Methods to determine significance of principal components is described in supplemental methods. The kinase signals that co-vary the greatest with the dependent variable (macrophage or osteoclast differentiation responses in this case) are weighted more heavily in the solution function that will be used to predict the responses (Y) from the given input data matrix (X). When analyzing biological measurements, these principal component axes can be assigned to biological phenomena such as differentiation, proliferation or apoptosis.

In the scores plot shown in figure 4D, treatment conditions are plotted onto principal component axes according to their covariance. In other words, similar cellular responses to treatments are grouped together when projected onto the principal components. PMA and 1,25D_3 stimulated kinase signatures were segregated along the first principle component (PC 1). Undifferentiated THP-1 monocytes cultured only in RPMI media were segregated along PC 2 from the differentiated cells. This suggested that the first principal component could be defined as macrophage/osteoclast differentiation axis, and the second as the proliferation axis. The loadings plot is shown in figure 4E and depicts the contribution of an individual kinases activation at a specific time, to macrophage or osteoclast differentiation according to the calculated weighted coefficients, and plots them onto weighted principal components. A clear polarization of signals with either CD68 or TRAP responses is depicted.

Goodness of prediction was tested using a bootstrapping approach; cross-validation

was performed by omitting an observation, then using the calculated weighted coefficient matrix to predict response values without those removed observations. This procedure was repeated until every observation had been excluded exactly once. Then predictability was determined using root mean square error between predicted and experimentally observed values. Using Akt, ERK1/2, p38, IκB, and JNK phosphorylation, greater than 99% predictability of CD68 expression and TRAP positive staining was achieved suggesting that these kinases would be useful in predicting monocyte differentiation responses.

3.3.2 Primary monocyte-derived macrophages and osteoclasts from healthy donors exhibit extensive morphological variability

The cell line was useful for proof-of-principle that a key set of kinase activation signatures could be predictive of monocyte differentiation decisions. The next step was to apply this methodology to primary monocytes isolated from different individuals peripheral blood. Monocytes were isolated from healthy donors and stimulated for macrophage differentiation with M-CSF for 14 days, or for osteoclast differentiation with M-CSF and RANKL for 14 days. Morphological differences in diameter and number of nuclei among the donors differentiated macrophages and osteoclasts were the first indicator of variability among donors. Representative images of differentiated cells are shown with donor matched macrophages and osteoclasts, by column (Figure 5A). Osteoclast differentiation was confirmed by multi-nucleation (n $\geq$3) and TRAP+ histological staining (Figure 5B).

Cell diameter and number of nuclei of monocyte derived macrophages and monocyte-derived osteoclasts were quantified and shown in the box and whisker plots (Figure 5C). Mean diameter of the macrophages was 29.2$\pm$12μm, 41% deviation in diameter, among seven donors, here on referred to as Group I, and with one nucleus. Osteoclasts exhibited greater range of donor variability with a mean diameter of 143.5$\pm$85μm, a 59% deviation, and median number of nuclei of 3 (Figure 5C) with one osteoclast

from a particular donor contained as many as 9 nuclei, although the median number of nuclei among all counted osteoclasts for that donor was 5. TRAP and CD68 expression were initially measured for first sets of samples using flow cytometry. However, changes in forward and side scatter (FSC and SSC) due to these morphology differences made it difficult to reliably gate and quantify appreciable shifts in fluorescence for these groups. Due to this, cell size and number of nuclei were used as quantitative morphological measurements.

3.3.3 Donor-to-donor variability in cell morphology is encoded in temporal kinase activation states of the differentiating monocytes

We tested the hypothesis that a coefficient matrix could still be calculated from multivariate kinase activation to predict donor monocyte differentiation responses despite the donor-specific morphological variability. Cell lysates were collected on days 1, 3, 6 and 9 of primary monocyte differentiation and temporal kinase phosphorylation signatures were measured using Bioplex assays as described earlier. These kinase activation signatures were variable for Group I, as indicated by color variations in the heat map, even for the same kinase, treatment condition, and time (Figure 6A). The input matrix (X) of kinase phosphorylation and dependent response matrix (Y) of cell morphology data of diameter and number of nuclei were used to calculate the weighted coefficient matrix that would link the initiating cues to the differentiated cell phenotype responses using the kinases signals. Separate models were made for macrophages and osteoclast differentiation cues, with the inherent, but unknown donor-specific factors that stimulate monocytes in vivo, affecting kinase signatures of both datasets.

PLSR analysis also can identify the most important kinase signals and time points for a differentiation outcome by calculating the variable importance for projection (VIP) using a weighted sum of squares of the coefficients calculated for a signal, such

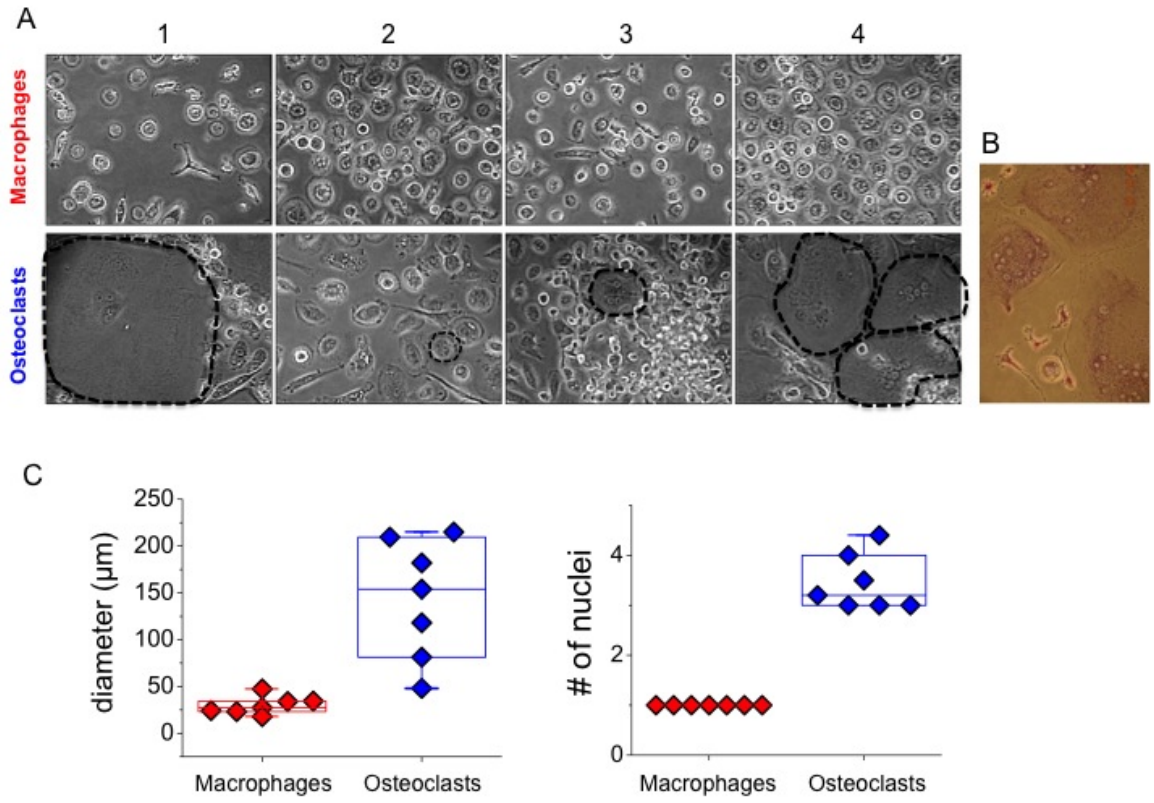

Figure 5: Phenotypic variability of macrophages and osteoclasts derived from peripheral blood monocytes Monocytes isolated from peripheral blood were cultured for 14 days and treated with 30ng/μl M-CSF alone to differentiate them into macrophages or with 30ng/μl M-CSF and 30ng/μl sRANKL to drive osteoclastic differentiation and cultured. A) Representative pictures of monocyte derived macrophages and monocyte derived osteoclasts after 12 days of differentiation are shown. Dotted lines outline osteoclasts. B) On day 15, multinucleated cells in the culture were stained for TRAP activity to confirm osteoclastic differentiation. A representative image of colorimetric assay staining is shown. C) Mean diameter and average number of nuclei per donor were measured (n=7).

that those signals projecting strongly either positively or negatively with a differentiation response are highly ranked. If the VIP value was greater than 1, then the kinase signals were regarded as significant. JNK activation on days 1, 3 and 6 were important for determining cell morphological responses for both macrophages and osteoclasts (Table **??**. Interestingly, the effects of JNK activation on macrophage and osteoclasts were opposite; with a positive correlation for macrophage diameter but a negative correlation for osteoclast diameter and number of nuclei. Using the bootstrapping method described earlier, goodness of prediction was calculated. Predictability was 97% for cell diameter of macrophages, and 93% for osteoclasts. Predictability was 95% for number of nuclei for osteoclasts (Figure 6B). With only one nucleus, no variation could be predicted for monocyte-derived macrophages.

To test this predictability a priori, this trained model was applied to 7 additional donors, denoted as Group II, with kinase activation heat map and variable signals shown (Figure 7A). Cell diameter was predicted at 90% and 92% for macrophages and osteoclasts, respectively, but osteoclast nuclei predictability dropped to 71% (Figure7B). Quantification of these experimental measurements for Group II is shown in box and whisker plots with mean diameter of the macrophages as 34.4 10μm (29% deviation) among 7 donors (Group II) and osteoclasts at a mean diameter of 173.9 54μm (31% deviation). Median number of nuclei for these osteoclasts was 4, one higher than training Group I, but two donors from Group II had higher average numbers of nuclei (>6) which could have lowered the nuclei predictability (Figure7C).

3.3.4 Cathepsin proteolytic activity of differentiated macrophages and osteoclasts reflect donor-to-donor variability

With some predictability of morphology, we tested if behavioral or functional activity of the differentiated macrophages and osteoclasts could also be predicted for individual donors. Cathepsin activity was this metric. After the 14 day differentiation period during which kinase phosphorylation data was collected, conditioned media and cell

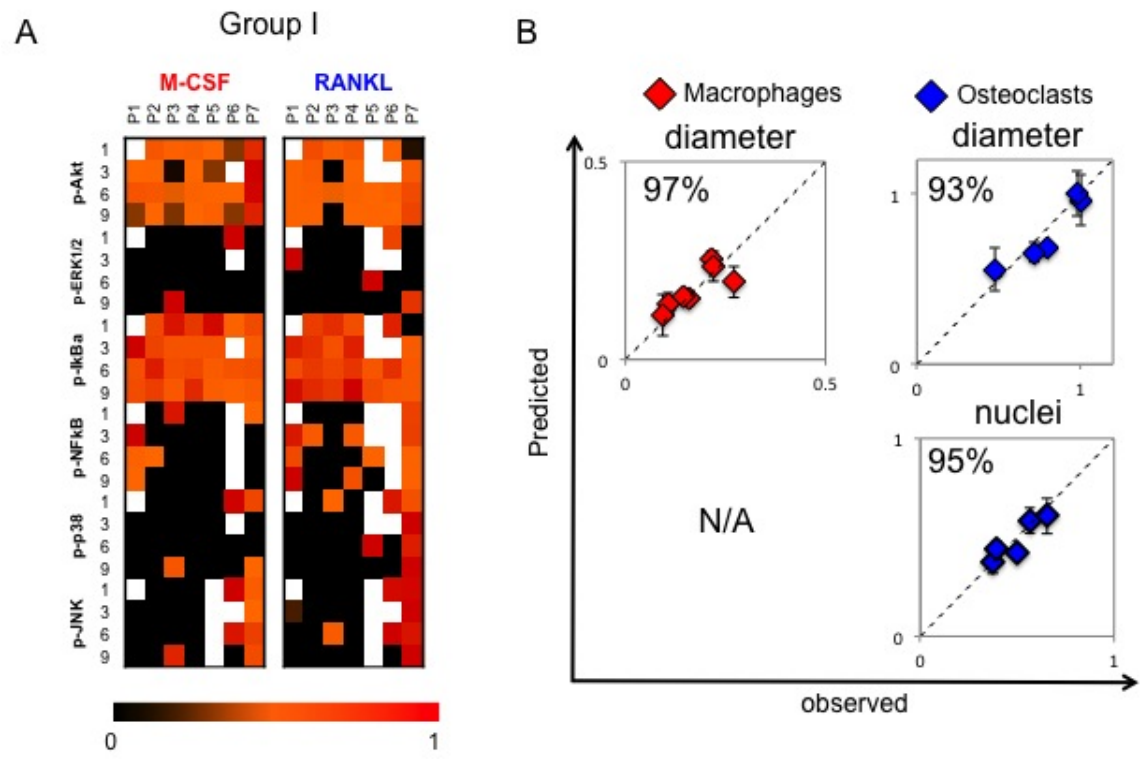

Figure 6: Donor-to-donor variability in macrophage and osteoclast differentiation outcomes of cell diameter and number of nuclei is captured in kinase activation state of differentiating monocytes A) On days 1, 3, 6 and 9 of differentiation, macrophages and osteoclasts were lysed, and kinase signals were quantified using Bioplex technology. A compendium of time-dependent kinase signals of donors 1-7 (group I) is shown. Signals are normalized to the highest value within a given time point and a kinase. White boxes correspond to missing measurements. B) A PLSR model was generated with kinase signals of differentiating macrophages as inputs and cell diameter as an output ($R^2Y = 0.734$ $Q^2 = 0.061$, 1 significant PC). A separate PLSR model was generated for differentiating osteoclasts with their kinase signals in input matrix and their cell size and number of nuclei in output matrix ($R^2Y = 0.815$, $Q^2 = 0.248$, 2 significant PCs). Prediction was made with cross-validation and jack-knifing approaches, and predictability was calculated based on RMSEE. Plots of predicted vs. observed are shown, with blue diamonds for osteoclasts and red diamonds for macrophages. Predictability for cell diameter was 96% for macrophages and 89% for osteoclasts. Predictability for number of nuclei was 94% for osteoclasts. With only one nucleus, no variation can be predicted for macrophage.

Table 1: **Variable importance of projection (VIP) for macrophage diameter and osteoclast diameter and number of nuclei** Kinase signals with significant VIP values for the PLSR model predictive of macrophage diameter, and osteoclast diameter and number of nuclei. Kinase signals were regarded as significant if the VIP value was greater than 1. Kinase signals with significant VIP values for both macrophages and osteoclasts were highlighted.

Macrophages		**Osteoclast**	
Variables	**VIP**	**Variables**	**VIP**
p-IkBa D6	1.550	**p-c-Jun day 6**	**1.349**
p-c-Jun day 1	**1.517**	**p-c-Jun day 3**	**1.243**
p-Akt day 1	1.478	**p-c-Jun day 9**	**1.182**
p-c-Jun day 3	**1.360**	p-IkBa day 6	1.152
p-IkBa day 1	1.073	p-Akt day 3	1.103
p-JNK day 9	1.063	p-Akt day 6	1.031
p-IkBa day 3	0.933	p-JNK day 1	1.018
p-IkBa day 9	0.865	p-JNK day 9	0.993
p-JNK day 6	0.842	p-IkBa day 3	0.932
p-Akt day 9	0.816	p-IkBa day 1	0.908
p-JNK day 1	0.746	p-Akt day 9	0.879
p-c-Jun day 9	0.667	p-c-Jun day 1	0.878
p-c-Jun day 6	0.665	p-Akt day 1	0.836
p-Akt day 3	0.496	p-p38 MAPK day 1	0.762
p-p38 MAPK day 1	0.419	p-JNK day 6	0.761
p-Akt day 6	0.345	p-IkBa day 9	0.714

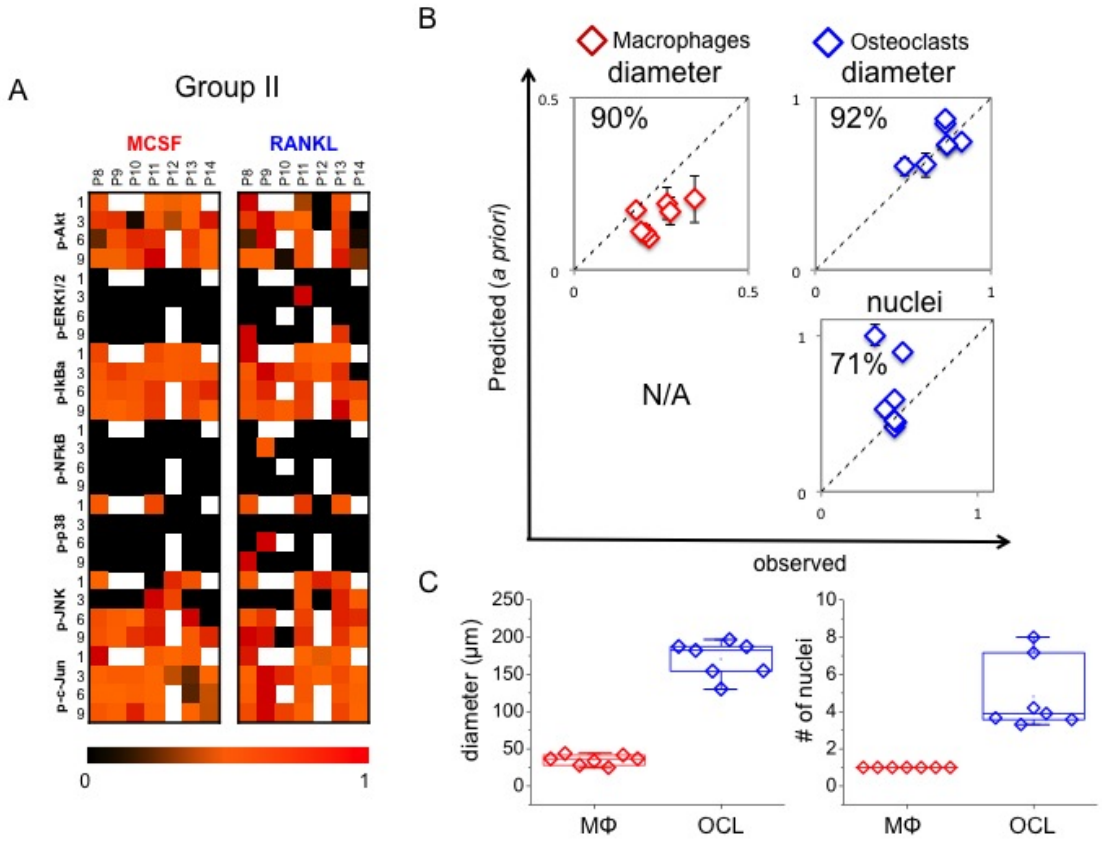

Figure 7: A priori predictions of differentiated morphologies A) A compendium of time-dependent kinase signals of donors 8-14 (group II) used for a priori prediction is shown. B) The trained models for macrophages and osteoclasts generated with Group I data were used to predict cell diameter and number of nuclei from Group II kinase data. The model was effective in predicting cell diameter, with 90% predictability for macrophages and 91% predictability for osteoclasts, but only 71% for osteoclast nuclei. With only one nucleus, no variation can be predicted for macrophage. C) Experimental/observed quantification of mean diameter and average number of nuclei per donor (n=7).

extracts were collected and assayed for cathepsin activity from a separate group of cells seeded from Group II donors. Multiplex cathepsin zymography was used. By this assay, active cathepsins K, L, S, and V produce cleared white bands of degraded gelatin on a Coomassie stained gelatin-polyacrylamide gel. Intensity correlates to level of proteolytic activity and this can be quantified with densitometry. An added benefit of this assay is that cathepsins K, L, S, and V all produce a detectable signal on the same gel but appear at distinct, expected electrophoretic migration distances of 37 kD for cathepsin K, 35 kD for cathepsin V, 25 kD for cathepsin S, and 20 kD for cathepsin L.[82] It is also more sensitive for cathepsin K than Western blotting.[105]

Cathepsin activity from cell extracts (Figure 8A,B) and conditioned media (Figure 8C,D) was quantified, and donor variability is shown in the box and whisker plots with representative gels of four donors zymograms shown. Zymograms shown in figure 5 were incubated at pH 4 for maximal cathepsin V and L signals, and were also incubated at pH 6 for maximal cathepsin K signal[202] (Figure 9). From these zymogram results, there were similar cathepsin activity profiles across donors within a cell type, but distinctive between osteoclasts and macrophages. A 75 kD cathepsin activity band consistently appeared in osteoclast lysates and conditioned media, suggestive of it being cathepsin K activity. However, its electrophoretic migration distance differed from the expected 37 kD distance for cathepsin K.[94] To verify its identity, osteoclast lysates were loaded for cathepsin zymography and incubated in the presence or absence of 1μM cathepsin K inhibitor, which blocked the appearance of the band in question after staining the zymogram, confirming its identity as cathepsin K (Figure 8E,F). Cathepsins K and V activity was higher in both conditioned media and cell extracts of osteoclasts compared to macrophages. This was expected for cathepsin K since it is the key enzyme used by osteoclasts for bone resorption.[23] Cathepsin V, however, was more unexpected since its tissue localization has been reported as being restricted to thymus, testis, cornea, and macrophages.[208] Macrophage cathepsin L

activity was high in both conditioned media and cell extracts supporting reports of its expression by macrophages in atherosclerosis.[115] Cathepsin L was not secreted by osteoclasts, however, although it was active intracellularly. Cathepsin L also showed the greatest donor-to-donor variability.

3.3.5 Donor-specific cathepsin proteolytic activity of monocyte-derived macrophages and osteoclasts can be predicted

To test predictability of cathepsins K, L, S and V activity in differentiated macrophages and osteoclasts, cell-type specific coefficient matrices were calculated using the previously collected kinase signatures from Group II. Predictability was calculated as before with cell diameter and nuclei data, but this time for cathepsin activity in cell extracts. Plots of predicted values versus experimentally observed values are shown in (Figure10). Predictability for all cathepsins was greater than or equal to 90%, and this was true for both macrophage and osteoclast outcomes. For macrophages, predictability for cathepsin K was 90%, cathepsin V was 95%, cathepsin S was 94%, and cathepsin L was 93% (Figure10A). For osteoclasts, predictability for cathepsin K was 90%, cathepsin V was 90%, cathepsin S was 95%, and cathepsin L was 90% (Figure10B). Predictions for cathepsins secreted into the conditioned medium were generally lower than 90% and considered not to be predictive (Figure 11). Analysis of the VIPs for predicting cathepsin activity identified c-jun phosphorylation as important for cathepsin activity of monocyte-derived macrophages and osteoclasts (Supp Table 2), and this has been implicated previously by us.[82]

3.4 Discussion

Variability in disease may be due to a number of factors. From these results, we submit that each persons individual biochemical milieu of cytokines, growth factors, and other stimuli, contain a bevy of cues that explicitly and acutely pre-condition cells for specific responses to induce the variability in response to treatment or in progression

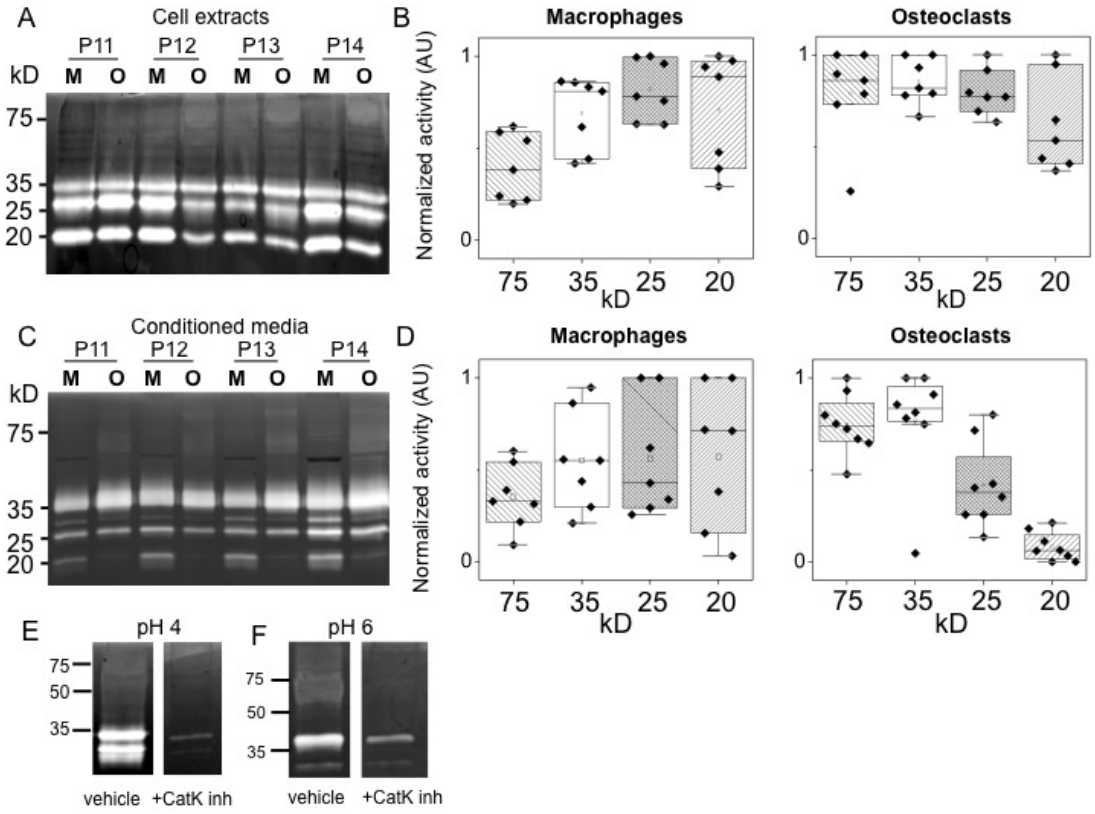

Figure 8: Cathepsin proteolytic profiles of differentiated macrophages and osteoclasts reflect donor-to-donor variability Multiplex cathepsin zymography (assay buffer, pH 4) and quantification of (A, B) cell extracts or (C, D) conditioned media for macrophage and osteoclast differentiation from donor monocytes. Quantification of cathepsin activity and donor variability is represented in the box and whisker plots. To confirm identity of 75kD band as cathepsin K, osteoclast lysates were loaded for zymography and 1μM of cathepsin K inhibitor (1-(N-benzyloxycarbonyl-leucyl)-5-(N-Boc-phenylalanyl- leucyl) carbohydrazide [Z-L-NHNHCONHNH-LF-Boc] was incubated with the zymogram during the overnight incubation at pH 4 (E) or pH 6 (F). The 75kD active band no longer appears.

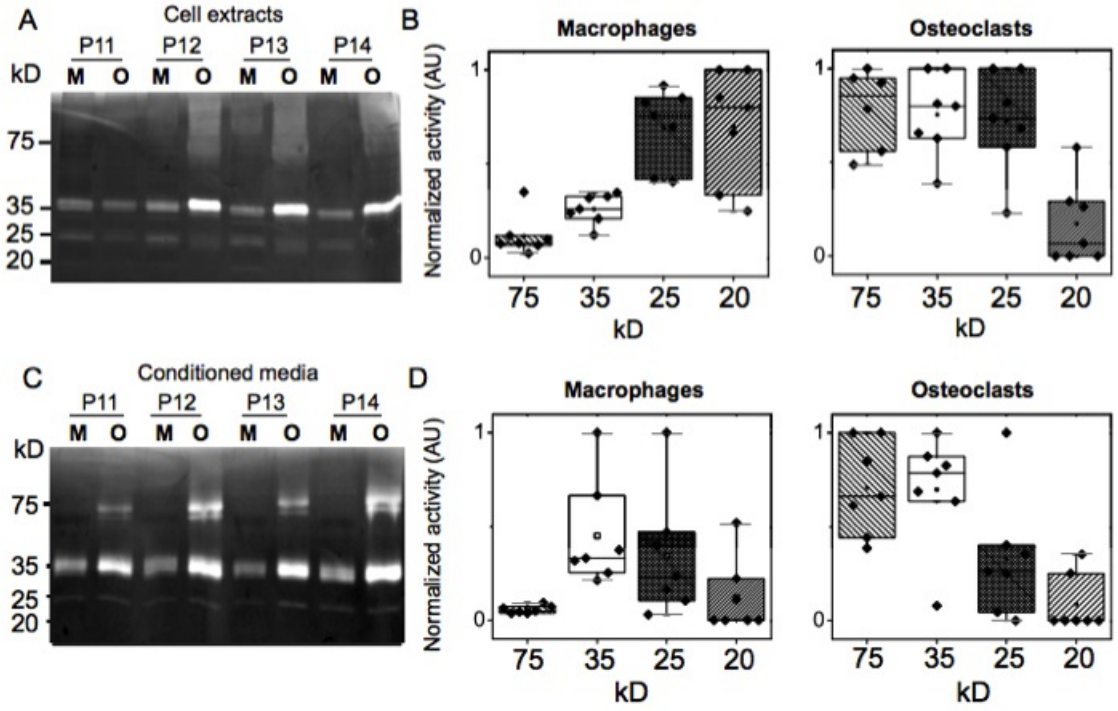

Figure 9: Cathepsin proteolytic profiles of differentiated macrophages and osteoclasts in assay buffer, pH 6. Multiplex cathepsin zymography (assay buffer, pH 6) and quantification of (A, B) cell extracts or (C, D) conditioned media for macrophage and osteoclast differentiation from donor monocytes. Quantification of cathepsin activity and donor variability is represented in the box and whisker plots. As with zymograms incubated in pH 4, the 75kD cathepsin activity was apparent in osteoclasts and cathepsin V activity was higher in osteoclasts as well. Cathepsin L activity was higher in cell extracts of macrophages.

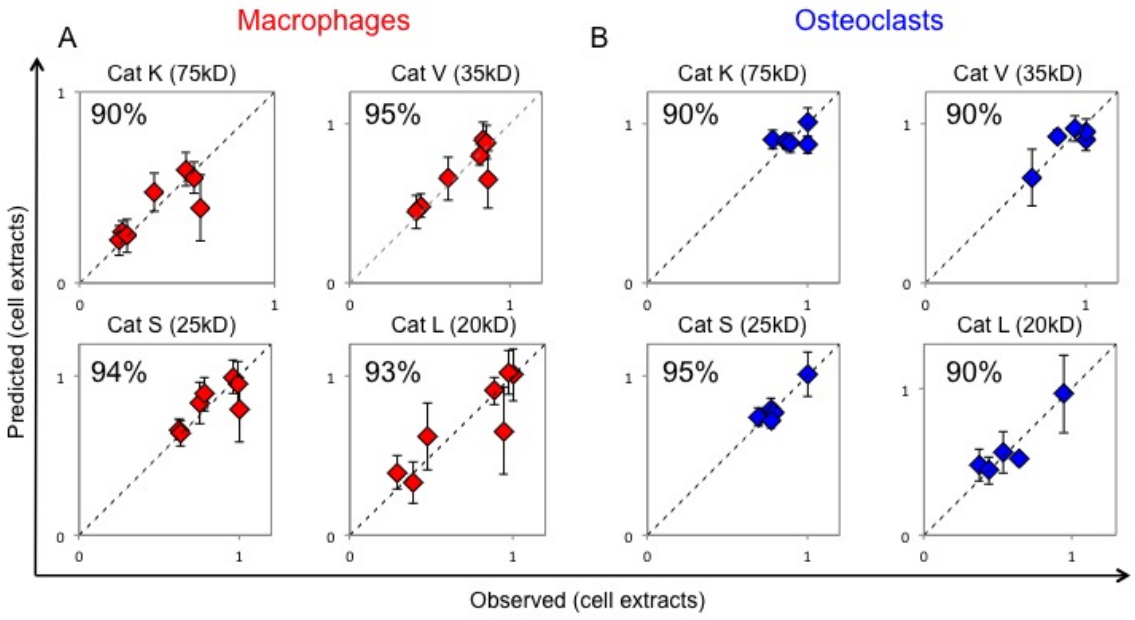

Figure 10: Multivariate analysis of kinase activation successfully predicted donor-specific cathepsin proteolytic activity of monocyte derived macrophages and osteoclasts Predictability of cathepsins K, L, S, and V activity in cell extracts using a PLSR model trained with kinase measurements of differentiating macrophages (A) ($R^2Y = 0.837$, $Q^2 = 0.618$, 1 significant PC) or of differentiating osteoclasts (B) ($R^2Y = 0.564$, $Q^2 = -0.0823$, 1 significant PC).

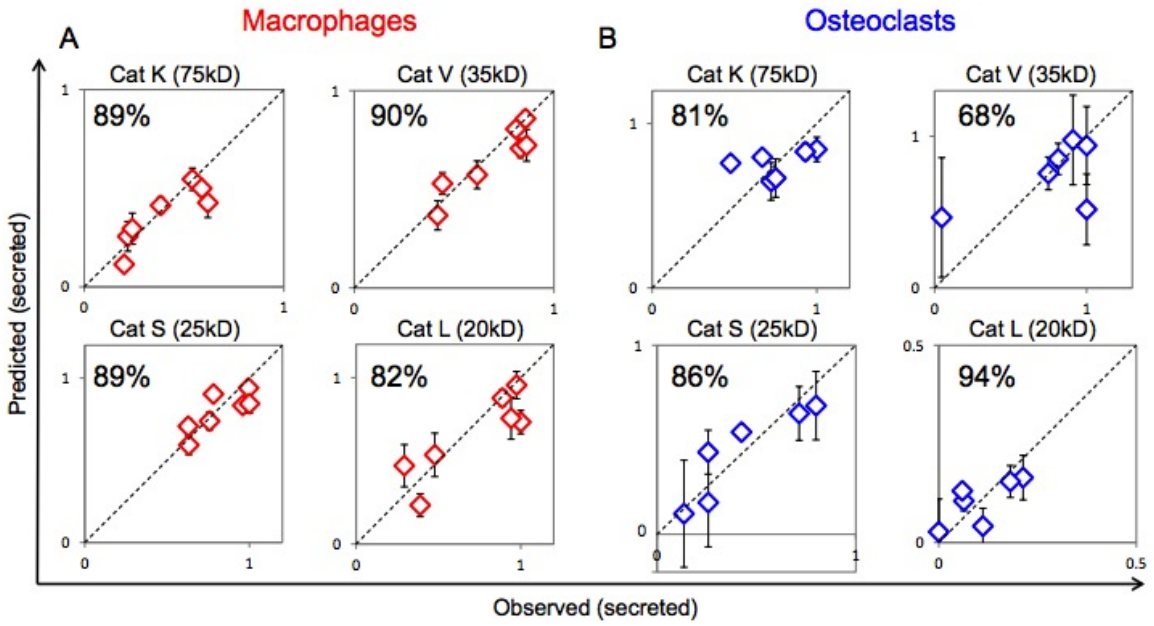

Figure 11: Multivariate analysis of kinase activation was not highly predictive of secreted cathepsin proteolytic activity of donor monocyte derived macrophages and osteoclasts. Predictability of secreted cathepsins K, L, S, and V activity using a PLSR model trained with kinase measurements of differentiating macrophages (A) ($R^2Y = 0.693$, $Q^2 = 0.541$, 1 significant PC) or of differentiating osteoclasts (B) ($R^2Y = 0.478$, $Q^2 = 0.073$, 1 significant PC).

Table 2: **VIPs for cell-associated cathepsin activity of macrophages and osteoclasts** Kinase signals with significant VIP values for the PLSR model predictive of cell-associated cathepsin activity of monocyte-derived macrophages and for monocyte-derived osteoclasts.

Macrophages		**Osteoclasts**	
Variables	**VIP**	**Variables**	**VIP**
p-JNK day 6	**1.782**	**p-JNK day 1**	1.710
p-JNK day 3	**1.596**	p-Akt day 1	1.685
p-JNK day 1	**1.439**	**p-JNK day 6**	1.593
p-NFkB p65 day 1	1.292	p-p38 MAPK day 6	1.207
p-ERK1/2 day 9	1.086	p-ERK1/2 day 6	1.189
p-p38 MAPK day 9	1.044	p-p38 MAPK day 1	1.162
p-IkBa day 3	0.918	p-ERK1/2 day 1	1.100
p-IkBa day 9	0.875	p-NFkB p65 day 1	1.096
p-NFkB p65 day 6	0.869	p-IkBa day 1	1.092
p-Akt day 9	0.800	p-IkBa day 9	1.065
p-JNK day 9	0.751	p-Akt day 6	1.058
p-Akt day 3	0.746	**p-JNK day 3**	1.053
p-p38 MAPK day 1	0.669	p-p38 MAPK day 3	1.053
p-IkBa day 6	0.586	p-Akt day 9	1.035
p-Akt day 6	0.505	p-NFkB p65 day 9	0.781
p-Akt day 1	0.272	p-JNK day 9	0.710
p-IkBa day 1	0.156	p-Akt day 3	0.684
		p-IkBa day 3	0.536
		p-NFkB p65 day 6	0.397
		p-IkBa day 6	0.316
		p-ERK1/2 day 3	0.285
		p-NFkB p65 day 3	0.277
		p-ERK1/2 day 9	0.156
		p-p38 MAPK day 9	0.156

of disease. Cathepsins are proteases expressed by macrophages and osteoclasts that are also biomarkers and mediators of tissue destructive diseases. By using a systems biology approach to link cell differentiation cues and responses through integration of signals at the kinase level, where integration of ubiquitous information is processed intelligently by the differentiating cell, we were able to mathematically predict relative amounts of cathepsin activity and distinguish which donors would have greater cathepsin activity compared to others.

Despite stimulation with identical cues, in vitro, donor-to-donor variability was evident in kinase activation signatures, differentiated cell morphology, and cathepsin activity. This suggests that there were additional cues that pre-conditioned the circulating monocytes from unique, donor-specific milieus prior to isolation. Along with a donors genetic background, there are many bioactive molecules circulating in the blood that control macrophage and osteoclast differentiation, such as monocyte chemoattractant protein-1, RANKL, tumor necrosis factor-, parathyroid hormone, and calcitonin, among others, that differ from donor to donor. The complex question of how to account for the influence of these undetermined and unmeasured cues may be answered using kinase signatures, as demonstrated by this study. Kinases are up- and downstream of growth factor binding to receptors, cytokine stimulation, transcription factor binding, gene transcription, and protein translation. Therefore, by measuring kinase activation as this nexus of inputs that precede outputs, predictive information of cell fate prior to synthesis of differentiated cell-specific proteins and behavioral responses is provided. Although there was variability in magnitude and duration of kinase activation signals among the seven original donors of this study, a weighted coefficient matrix could be used to calculate and predict the responses of cell diameter and number of nuclei with as high as 97% predictability (Figure 6). Osteoclasts larger cell diameters and multi-nucleation are distinct characteristics from macrophages, although they differentiate from the same progenitor cell. By using

these metrics, PLSR was able to predict differentiation outcomes in terms of cell diameter and number of nuclei. Even further, it was predictive of the donor-specific differences in these outcomes, which varied by as much as 59% for osteoclasts (Figs 3 and 4).

This was tested a priori on a second group of donors with predictability remaining above 90% for cell diameter, but an additional functional outcome of cathepsin activity was measured to link the donor variability in differentiated macrophages and osteoclasts to cell function; cathepsin activity from cell extracts was also highly predictive using only temporal kinase signatures (Figure 10), and macrophage models were more predictive than osteoclasts. This is a limitation of the model, but it makes sense considering the wider range of values for osteoclasts and the fact that they are multi-nucleated cells formed from fusion of pre-osteoclasts. Another limitation is that it was difficult to predict secreted cathepsin activity levels in a donor specific manner (Figure 11). As an explanation, cathepsins are highly regulated at multiple stages from transcription to translation to secretion, and are susceptible to external influences such as degradation, oxidation, denaturation, and inhibition.[16] Additionally, we have recently shown that cannibalism occurs between cathepsins in the extracellular space serving to degrade each other,[7] and the time frame at which conditioned media is collected could allow this additional behavior to occur and reduce the amount of secreted cathepsin present. These factors cannot be accounted for by changes in intracellular kinases. The multivariate analysis was able to provide clues to identify cathepsin K despite its altered electrophoretic migration. Cathepsin K is post-translationally modified in several ways that can affect its electrophoretic migration under non-reducing conditions: 1) its glycosylation can lead to altered targeting and secretion,[16] 2) it binds to chondroitin sulfates to form large oligomeric complexes,[107, 106, 108] and 3) there are also reports of cathepsin K assuming either a tensed or relaxed state depending on ionic conditions at physiological pH that may

be altering its electrophoretic migration.[149]

Development of cathepsins as biomarkers of disease is a growing field, but the lack of predictability for secreted cathepsins shown in this study, may have translated to the difficulties of others that have used ELISAs to measure cathepsins in plasma or serum and correlated their levels with disease. There have been varied successes in cardiovascular disease, osteoporosis, cancer, and osteolytic bone metastases.[113, 96, 94, 84, 2] Serum cathepsin K measurements have been controversial; one study determined statistically significant elevated cathepsin K in post-menopausal women with osteoporosis compared to healthy age matched women, even though the standard deviation of the measurements was greater than the mean.[96] A different study reported the opposite; serum cathepsin K levels could not be used to identify pre- and post-menopausal women with osteoporosis or osteopenia.[96] In cancer biomarker studies, serum cathepsin levels have been measured for prostate, breast, and lung cancer, and all have yielded wide range of donor to donor variability and inconclusive findings regarding their correlation with disease.[96, 191, 145] By studying mononuclear cells from the blood and the proteases they will produce, inferences can be made about local proteolytic activity contributing to the focal disease and matrix degradation, whereas the circulating levels in the blood could be attributed to any number of cells from different regions of the body. Those large cohort studies motivate the need for personalized medicine approaches for individualized assessment incorporating the indefinable, therefore non-quantifiable patient-inherent factors that provide cues to elicit cellular responses; these factors may explain why some individuals have greater propensity to make proteolytic enzymes over others (Figure 8). In this study, monocytes were specifically targeted as they are the effector cells that enter tissue, differentiate, and advance disease. Recently, the effects of some inherent, circulating donor-specific factor (or a group of factors) that increased protease production by monocytes was demonstrated in a comparative study between individuals with

and without sickle cell disease.[82] In that study, we showed that the chronic inflammatory milieu of sickle cell disease activated monocytes to induce greater cathepsin proteolytic activity after binding to endothelial cells compared to those without the disease. This could be an example of donor-inherent factors that precondition monocytes for elevated proteolytic activity. Although the donors of this current study did not have sickle cell disease, they still exhibited wide range of variability in cathepsin activity, perhaps due to each persons unique biochemical milieu; yet kinase activation signals could predict these responses.

Donor variability in kinase activation signatures and cathepsin activity profiles may provide insight into proper dosing and efficiency of therapeutic small-molecule kinase inhibitors and cathepsin inhibitors currently in the pharmaceutical pipeline. Variability among the donors kinase activation (Figure 6A, 7A) also suggests that a one-size-fits-all approach of administering kinase inhibitors may not be the best strategy for all patients. Certainly the varying levels of active cathepsins among patients can be a confounding factor when prescribing doses of cathepsin inhibitors and cause severe side effects in some patients, that have prematurely ended many cathepsin S and K inhibitor clinical trials.[18]

3.5 Conclusions

Kinases are signal integrators between environmental cues and cellular responses, and analysis of multiple kinase pathways yielded high predictability for monocyte differentiation into macrophages and osteoclasts, described by cell morphology and cathepsin activity. Although there are a myriad of donor-specific factors that cannot be accounted for, we suggest that the wide range of donor-to-donor variability in proteolytic expression from monocyte-derived macrophages and osteoclast shown in our study may provide clues to wide range of disease progression and responses to therapy observed between patients. Lastly, using data-driven, multivariate analysis

model of kinases, we could predict donor-specific cathepsin activity profiles which may provide beneficial tools for personalized protease inhibitor therapies.

CHAPTER IV

DONOR-TO-DONOR VARIABILITY IN MACROPHAGE- AND CATHEPSIN-MEDIATED BREAST CANCER CELL INVASION

4.1 Introduction

Despite the prevalence of breast cancer in the U.S.[142], patient-to-patient variability in disease progression continues to complicate clinical decisions in diagnosis and treatment of patients.[179, 152, 27, 40, 153, 180, 204] Advancement in diagnostic and imaging techniques led to increases in early detection of breast cancer. This increase, however, often leads to premature and aggressive treatment of non-malignant lesions due to inherent uncertainty in malignant progression of the cancer. A lack of well informed risk/benefit analysis can result in net harm to the patients. This current work turns to tumor microenvironment to identify potential patient-specific predictive markers for cancer progression.

One main player that promotes invasiveness of cancer cells is tumor-associated macrophages (TAMs).[58] TAMs have been shown to promote angiogenesis,[102, 103, 104, 37, 165, 131] tumor growth[103], invasion and metastasis[109] through secretion of cytokines to coordinate tumor-promoting immune responses[6, 175, 120, 121] as well as through secretion of tissue-remodeling cysteine cathepsin proteases.[56, 57, 138, 199, 167] Moreover, infiltration of TAMs is often associated with poor prognosis.[34, 97, 100, 22] Cathepsins secreted by TAMs have been shown to play significant role in cancer growth and invasion.[56]

Previously, we showed donor-to-donor variability in cathepsin activity from primary monocyte-derived macrophages. In this current study, we investigate whether

this variability in cathepsin activity leads to interdonor variability in macrophage-mediated cancer cell invasion. donor MDMs were co-cultured with MCF-7 breast cancer cell line, thereby limiting the variability in the system to donor-specific factors. We also seek to identify proteolytic or molecular signatures that could be useful in identifying those with macrophages with high cathepsin activity and invasive potential

4.2 Materials and Methods

4.2.1 Primary monocyte isolation and differentiation

Heparinized venous blood from healthy volunteers was diluted 1:1 in sterile PBS and layered on Ficoll-Paque (GE healthcare) and centrifuged at 400g for 30 minutes. The buffy coat was isolated, red blood cells lysed, and peripheral blood mononuclear cells (PBMCs) were washed 3 times in PBS. For monocyte phosphoprotein and cathepsin activity analysis, $CD14^+$ and $CD16^+$ monocytes were isolated using Pan-monocyte magnetic bead isolation kit (Milteny) and lysates were collected. For macrophage differentiation, monocytes adhered overnight were cultured in RPMI containing 10% male human serum and 30ng/μl macrophage colony stimulating factor (M-CSF, Peprotech). Media was replaced every 3 days. For kinase inhibition studies, differentiating monocytes were treated with either 50μM JNK inhibitor II (SP600125, Millipore) or 2.5μM LY294002 (PI3K inhibitor, Millipore) with media change every three days for 12 days.

4.2.2 Multiplex cathepsin zymography

Cell extracts from monocytes and cell extracts and conditioned media from macrophages were collected. To prepare conditioned media, differentiation media was replaced with serum-free media on day 14 and incubated overnight. Conditioned media was collected and concentrated using VivaSpin®500 Centrifugal Concentrator (Vivaproducts). Cellular protein was extracted in lysis buffer (20 nM Tris-HCl at pH 7.5, 5 mM

EGTA, 150 mM NaCl, 20 mM β-glycerol-phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.1% Tween-20) with 0.1 mM leupeptin freshly added. Cathepsin zymography was performed on cell extracts and on conditioned media as described previously.[202] Briefly, equal amounts of protein in non-reducing loading buffer were separated on 12.5% SDS-polyacrylamide gels containing 0.2% gelatin at 4 °C. Enzymes were renatured and then the gels were incubated overnight at 37 °C in phosphate buffer, pH6 with 1mM EDTA and freshly added 2mM DTT. Gels were then rinsed, stained with Coomassie blue, and imaged using an ImageQuant LAS 4000 (GE Healthcare). Densitometry was performed using ImageJ to quantify the intensity of the white cleared band of proteolytic activity

4.2.3 Measurements of cystatin C

Conditioned media collected from differentiated macrophages were loaded for Western blot or ELISA to measure cystatin C level using mouse monoclonal antibody against cystatin C (Santa Cruz Biotechnology) for Western blot and Quantikine Cystatin C ELISA kit (R&D Biosystems).

4.2.4 Collagen invasion assay

Collagen invasion assay was adopted from a previous work by Goswami et al.[59] On day 13, MDMs were stained using 25M CellTracker Blue CMAC (Invitrogen) for 90 minutes. Then MDMs (n=160,000) and MCF-7 cells (n = 64,000) were plated on a 12-well MatTek multiwall plates in RPMI with 10% human AB serum. After overnight incubation, cells were serum starved for 4 hours in serum-free RPMI. Then cells were overlaid with 1,000μm layer of 2.5 mg/mL collage I and was allowed to gel for 90 minutes at 37 C before adding 1mL of RPMI with 10% human AB serum. After 24 hour incubation, cells and the collagen gels were fixed with 10% neutral buffered formalin and analyzed by confocal microscopy. Optical z-sections were taken every 5μm from the bottom of the plate. MCF-7 Cells that had invaded into collagen gel

beyond 20μm were counted and were divided by the number of MCF-7 cells at the bottom of the plate

4.2.5 Kinase phosphorylation analysis

On days 0, 1, 3, 6 and 9, freshly isolated monocytes or differentiating cells stimulated with M-CSF were lysed and total protein was determined using BCA (Pierce). Bioplex bead kits (BioRad) were used according to manufacturers instructions with 5 g protein from each sample and measured phosphorylation of ERK1/2 (Thr^{202}/Tyr^{204}, Thr^{185}/Tyr^{187}), Akt (Ser^{473}), p38 MAPK (Thr^{180}/Tyr^{182}), JNK (Thr^{183}/Tyr^{185}), c-jun (Ser^{63}), and IκB-α (Ser^{32}/Ser^{36}). Signal values for each phosphorylated kinase were normalized to the signal detected in a master lysate prepared in bulk from pre-stimulated cells that was used as a control for all assays. Signal values for each kinase were normalized to between 0 and 1 by dividing by the maximum value over the entire 9 days for all treatments.

4.2.6 Partial least square regression analysis

M x N data matrix was generated with data from M donors and N kinase phosphorylation signals or N cathepsin activity and cystatin C level. Each column of the independent X matrix corresponds to a unique input or signal: phosphorylated kinase signal from days 0, 1, 3, 6, and 9, and each column of the dependent Y matrix corresponds to unique responses which were cathepsin activity, cystatin C level or invasion index. Each row represents a unique donor and stimulation condition (i.e. donor 1-DMSO, donor 1-JNK inhibitor, donor 1-PI3K inhibitor, etc.). All data was mean-centered and scaled to unit variance. SIMCA-P (UMetrics) was used to solve the PLSR problem with the nonlinear iterative partial least squares (NIPALS) algorithm.

4.3 Results

4.3.1 Donor-to-Donor variability in macrophage cathepsin activity and cystatin C level underlie interdonor variability in cancer cell invasion

In our previous study, we reported donor-to-donor variability in cathepsin activity of monocyte-derived macrophages. Because many studies have shown that tumor-associated macrophages and cathepsins promote cancer cell invasion, we investigated whether the variability in macrophage cathepsin activity we observed would lead to variability in macrophage-mediated cancer cell invasion. Monocytes were isolated from peripheral blood drawn from healthy donors using density gradient centrifugation and were stimulated with M-CSF for 14 days. On day 14, differentiated macrophages were plated onto a transwell coated with Matrigel with or without MCF-7 breast cancer cells for modified Boyden chamber assay. After 24 hours, the number of invaded cells was counted and invasion index was calculated. Invasion index was defined to be the ratio between the number of invaded cells in the co-culture system to the number of invaded cells in MCF-7-only culture. There was donor-to-donor variability in the number of invaded cancer cells (Fig 12A). Cancer cells co-cultured with macrophages from P3 invaded four times less than the cancer cells co-cultured with donor 4 and twice less than donor 2. As the cancer cells were from clonal population of MCF-7 breast cancer cell line, to determine whether the variability was due to cathepsin secreted by macrophages, conditioned media was collected from the differentiated macrophages after 14 days and an equal amount of protein was loaded for multiplex cathepsin zymography (Fig 12B). Although there was apparent variability in secreted cathepsin activity between donors, donor 3 who had the least invasion had the highest cathepsin activity and donor 4 and 5 with high number of invaded cells had low cathepsin activity. These results were in contradictory to studies that showed cathepsins secreted by macrophages promote cancer cell invasion. However, it was also shown that lack of cystatin C, an endogenous inhibitor of cathepsins, increases

cancer cell invasion. We therefore turn to measure the amount of cystatin C secreted by macrophages as studies have shown that lack of cystatin C can promote cancer cell invasion. [148] The amount of cystatin C in conditioned media was measured using Western blot and shown in figure 12C. As suspected, donor 3 who had the highest macrophage cathepsin activity but the least invasion also had the highest amount of cystatin C which could inhibit cathepsin activity in the system leading to lower invasion of cancer cells.

A study showed that activation of JNK/c-Jun[83] stimulates cathepsins activity. To investigate their role in mediating donor-specific differences in cathepsin activity and invasion, we collected monocyte lysates on days 1, 3, 6 and 9 and measured phosphorylation of six kinases: ERK1/2, Akt, p38 MAPK, JNK, c-jun and IkB-a. In order to identify kinase signals that contribute most significantly toward proteolytic and invasion outcomes, we trained a PLSR model where kinase signals were a signaling matrix (X) and the outcomes were the response matrix (Y). Based on calculation of VIP scores, phosphorylation of JNK/c-Jun and Akt was determined to be important in determinining the outcomes. Activation of Akt has been associated with either increase[86] or decrease[209] in cell motility and invasion.

4.3.2 JNK inhibition reduces macrophage cathepsin activity and cystatin C level specifically among the subset of donors with high cathepsin activity

Based on the VIP scores as well as on previous studies that suggested role of JNK activation in cathepsin activity, we tested the hypothesis that inhibiting JNK/c-Jun pathway will reduce macrophage cathepsin activity and subsequent invasion of cancer cells. Freshly isolated monocytes from 12 donors were treated with SP600126 (50μM, JNK inhibitor II, EMD Millipore) for 14 days. The inhibitor was added freshly with each media change on days 1, 3, 6 and 9. On day 14, differentiation media was replaced with serum free RPMI and lysates and conditioned media were collected after

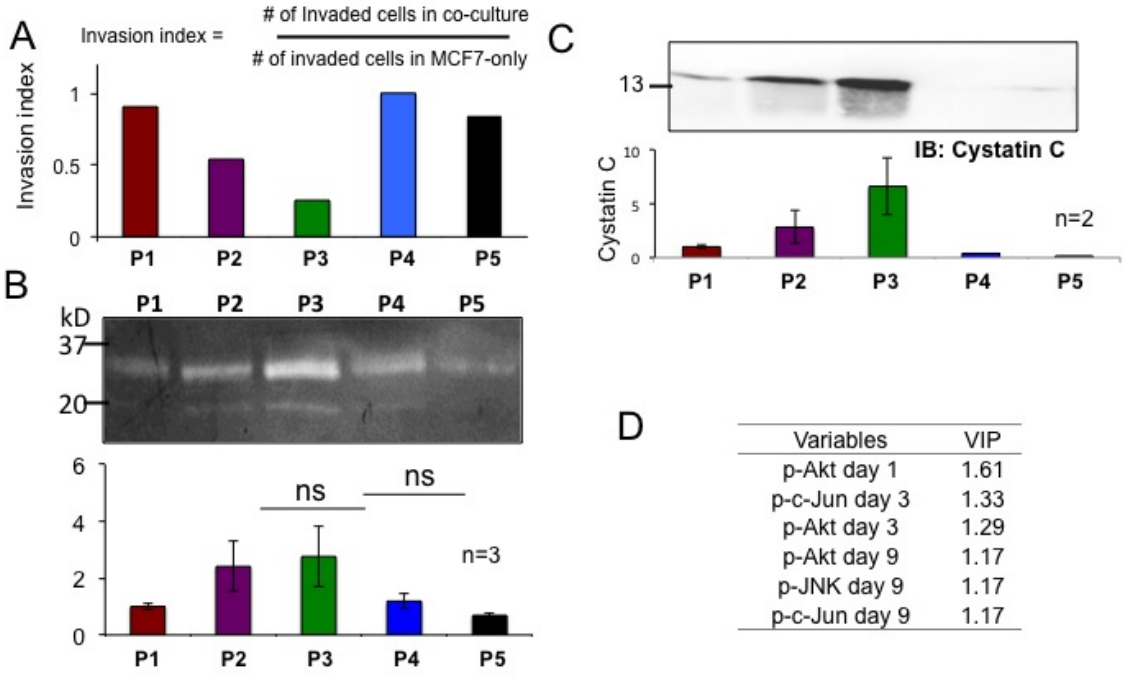

Figure 12: Interdonor variability in cancer cell invasion is mediated by donor-to-donor variability in macrophage cathepsin activity and cystatin C level (A) Monocytes isolated from five donors among the first set were stimulated with M-CSF for 14 days to differentiated them into macrophages. Conditioned media was collected from days 14-15 and loaded for cathepsin zymography to measure secreted cathepsin activity which was quantified through densitometry. (B) On day 14, differentiated macrophages were plated onto a transwell inserts coated with Matrigel with or without MCF-7 breast cancer cells. After 24 hours, number of invaded breast cancer cells were counted and invasion index was calculated. (C) The amount of cystatin C in conditioned media was measured using Western blotting. (D) On days 1, 3, 6 and 9, differentiating monocytes were lysed, and kinase signals were quantified using Bioplex technology.

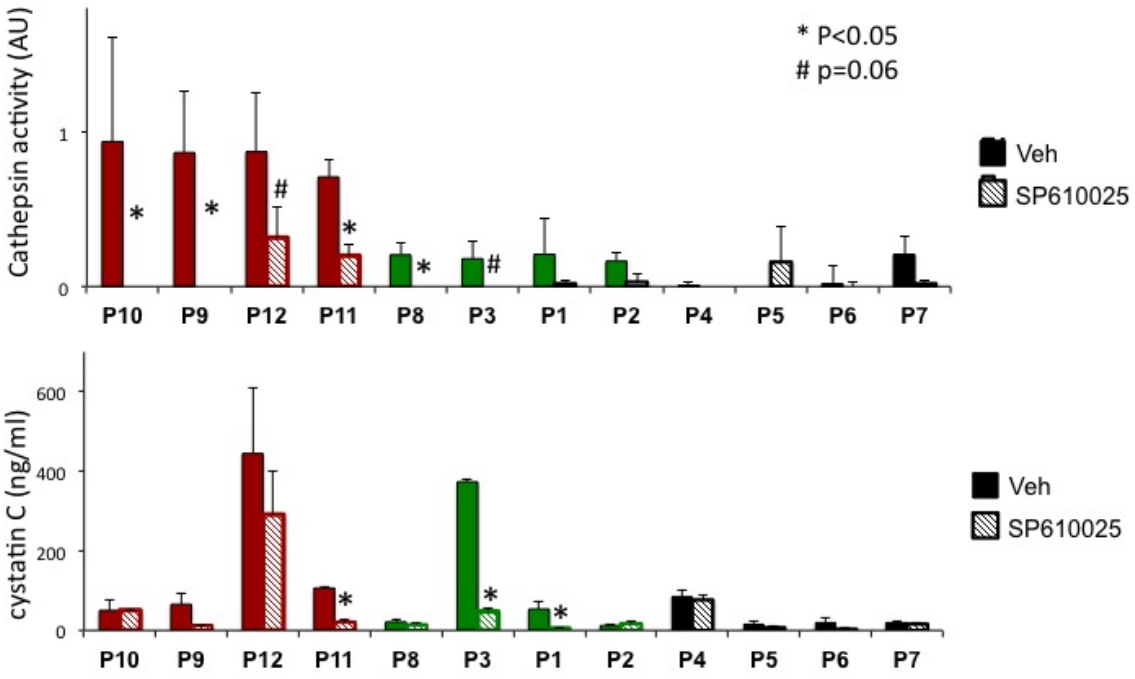

Figure 13: JNK inhibition reduces macrophage cathepsin activity and cystatin C level specifically among subset of donors with high cathepsin activity Freshly isolated monocytes from the same 5 donors were treated with SP600126 (50μM, JNK inhibitor II, EMD Milipore) or LY294002 (2.5μM, PI3K inhibitor, EMD Milipore) for 14 days. The inhibitor was added freshly with each media change on days 1, 3, 6 and 9. On day 14, differentiation media was replaced with serum free RPMI and lysates and conditioned media were collected after incubating cells for 14-16 hours. Cathepsin activity was measured using multiplex cathepsin zymography (A) and cystatin C level was measured with ELISA (R&D Systems) (B).

incubating cells for 14-16 hours. Cathepsin activity was measured using multiplex cathepsin zymography and cystatin C level was measured with ELISA (R&D Systems). There was a significant decrease in secreted cathepsin activity in macrophages from 4 out of 12 donors. Additionally, macrophages from 2 other donors showed a trend toward reduction in secreted cathepsin activity (Figure 13A). Interestingly, four of these donors had the highest cathepsin activity before inhibiting JNK. Cystatin C was reduced in macrophages isolated from donors 1, 3 and 11 (Figure 13B). No clear correlations were found between JNK regulation of cathepsin and cystatin C.

4.3.3 JNK inhibition reduced MDM-mediated cancer cell invasion for highly invasive donors

As was shown earlier, both cathepsin activity and cystatin C levels from macrophages influence cancer cell invasion. Therefore, we tested the hypothesis that the macrophages with high cathepsin activity or low cystatin C level will have the highest invasive potential. Differentiated macrophages treated with JNK inhibitors were co-cultured with MCF-7 breast cancer cells for collagen I invasion assay. After treating differentiating monocytes with JNK inhibitor for 13 days, as described earlier. On day 13, macrophages were stained with CellTracker Blue CMAC (Invitrogen) and plated with MCF-7 breast cancer cells. On day 14, following serum starvation for 4 hours, cells were overlayed with 2.5mg/ml collagen I (Invitrogen) and media was added. After 24 hours, the collagen discs were fixed and the percentage of breast cancer cells invading at least 20 μm into the collagen was determined by taking 5μm optical sections with a confocal microscope (Figure 15). Inhibiting JNK in differentiating macrophages reduced invasion of cancer cells co-cultured with macrophages from donors 8, 10 and 11. Their macrophages had the greatest invasive potential before JNK inhibition.

4.3.4 JNK inhibition reduces interdonor variability in macrophage cathepsin activity

Based on the results regarding donor-specific decreases in cathepsin activity after JNK inhibition, we re-examine the effects of JNK inhibition for all donors as one cohort, instead of for each donor. Population variance between the two groups were compared using Brown-Forsythe test and variance was shown to be significantly reduced after JNK inhibition (Figure 16 A). Variability in Cystatin C level was not affected by JNK inhibition, but there was slight reduction in cancer cell invasion. To test that this reduction in variance is specific to JNK inhibition, we inhibited Akt, another kinase with high VIP scores, in differentiating monocytes and measured cathepsin activity, cystatin C level and invasion. There was no significant change in the variance with

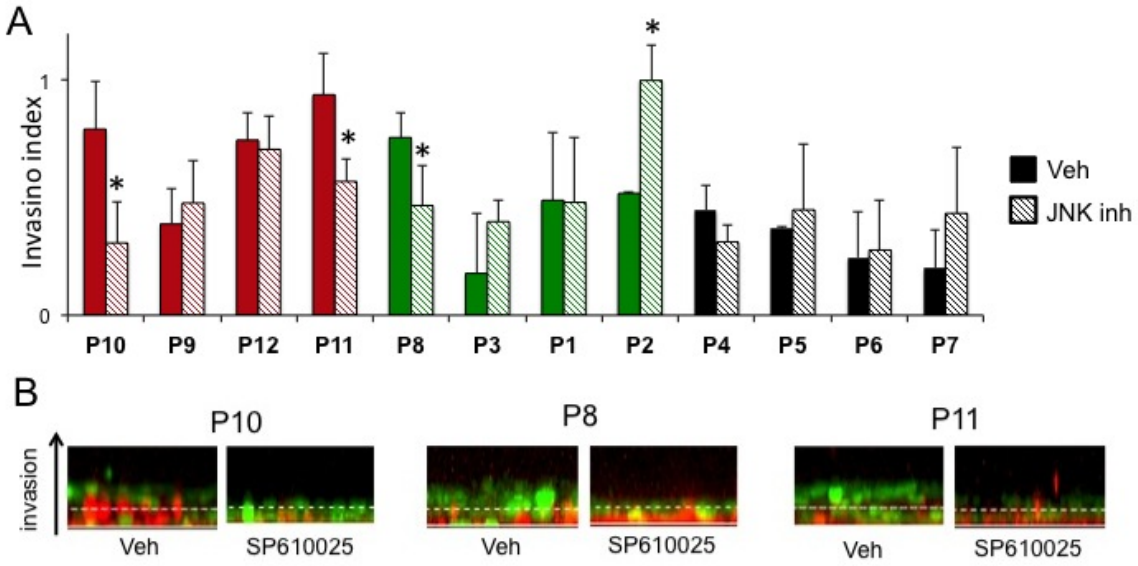

Figure 14: JNK inhibition significantly reduced MDM-mediated cancer cell invasion for highly invasive donors (A, B) After inhibiting JNK in differentiating monocytes for 12 days, monocyte-derived macrophages were co-cultured with MCF-7 breast cancer cell line for collagen I invasion assay. MDM-mediated cancer cell invasion was significantly reduced among the donors with highest invasion (donors 8, 10 and 11).

Akt inhibition.(Figures 16 B, C). A scores plot of PLSR model trained with cathepsin activity, cystatin C level and invasion shows clustering of macrophages treated with JNK inhibitors (Figure 16 D).

4.3.5 JNK inhibition selectively reduces cathepsin activity and invasion for donors with JNK^{low} monocytes

Because JNK inhibition reduced macrophage cathepsin activity and cancer cell invasion among the donors with high cathepsin activity, we investigated if there are distinguishing characteristics of circulating monocytes that can be identified without differentiating them into macrophages for multiple days. We hypothesized that the donor macrophages with higher cathepsin activity will have high monocyte cathepsin activity and JNK activation. To test this, we isolated monocytes from all 12 donors using magnetic activated cell sorting and lysed the cells to measure their cathepsin activity and activated kinase signals. Monocytes isolated from donors 8, 9, 11 and 12 had high cathepsin activity but no detectable amount of activated JNK (Figure 16A). P10 also had no detectable amount of activated JNK but also no monocyte cathepsin activity. These results suggest although there is no apparent correlation between monocyte and macrophage cathepsin activity, for the donors with p-JNK^{low} monocytes, inhibiting JNK in macrophages reduces cathepsin activity as well as macrophage-mediated cancer cell invasion.

4.4 Discussion

In the current study, we show that interdonor variability in cancer cell invasion is associated with the donor variability in macrophage cathepsin activity and cystatin C level. Although we acknowledge that the tumor microenvironment is extremely diverse in terms of cellular population, cytokines, and growth factors, we were able to isolate the effects of variability in donor macrophage phenotype on cancer cell invasion by creating an *in vitro* model of cancer cell invasion where these facets can

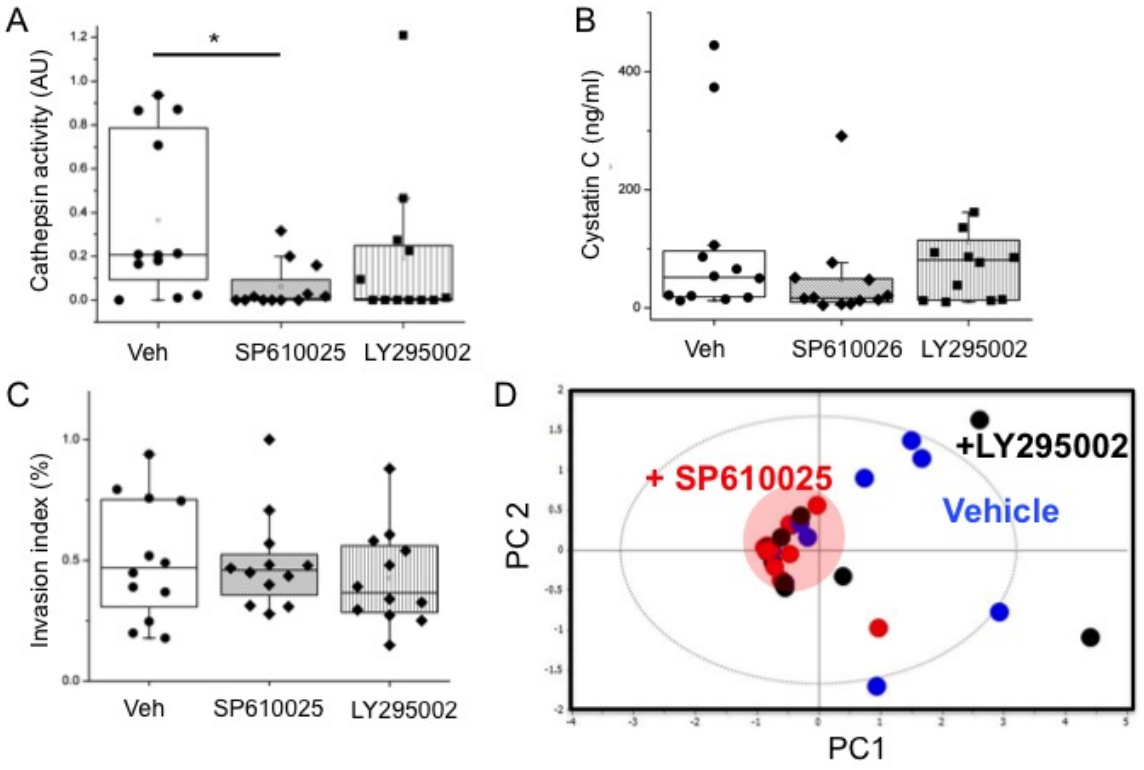

Figure 15: JNK inhibition reduces donor-to-donor variability in macrophage cathepsin activity (A) When macrophage cathepsin activity, cystatin C levels and cancer cell invasion were compared between treatment groups for the entire donor cohort, there was significant reduction in macrophage cathepsin activity after JNK inhibition as well as significant different in variance between the two groups. PI3K did not reduce cathepsin activity significantly (B, C) Inhibiting JNK or PI3K did not reduce cystatin C level or invasion significantly for the entire cohort. (D) A PLSR scores plot for a model trained with cathepsin and cystatin C level as signals and invasion as a response depicts the reduction in donor variability in macrophage phenotype upon JNK inhibition

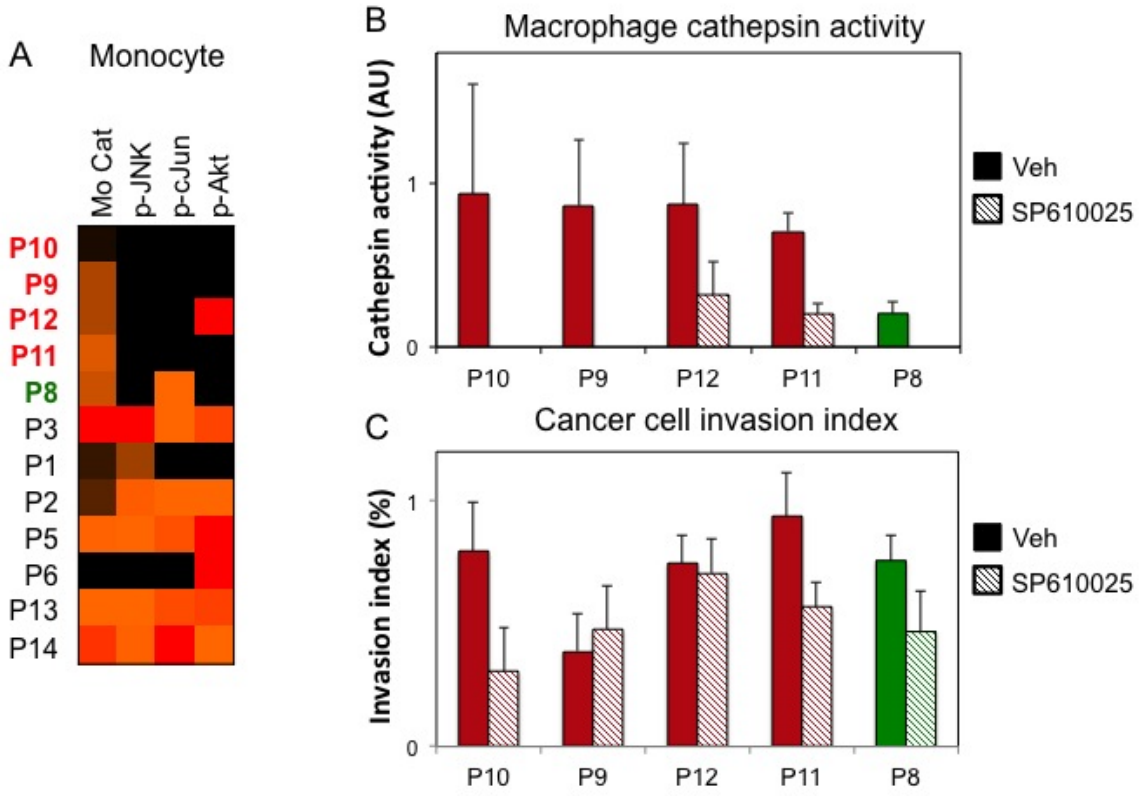

Figure 16: JNK inhibition reduces macrophage cathepsin activity and cancer cell invasion among donors with p-JNK^{low} monocytes (A) Freshly isolated monocytes were lysed for multiplex cathepsin zymography or Bioplex kinase assays to measure cathepsin activity or kinase activation. Three kinases that were important for macrophage cathepsin activity are shown in the heat map. (B, C) Cathepsin activity and invasion was reduced significantly among donors 8, 9, 10, 11 with low to undetectable level of activated JNK.

be controlled and limited.When the effects of JNK inhibition were examined across all the donors as one cohort, only cathepsin activity was shown to be decreased, but other donor-specific responses for cystatins and invasion were masked by the population variance. However, upon examining individual responses, we were also able to reduce invasion among donors whose macrophages have high invasive potential by inhibiting JNK/c-Jun pathway in differentiating monocytes. Notably, inhibiting JNK increased invasive potential for macrophages from donor 2. This finding may have important implications for personalized medicine approaches as although donor cohort studies reveal important mechanistic information, each donor, especially those who are outliers need to be examined individually for accurate diagnosis or treatment. Moreover, with the increased development of small molecule inhibitors to treat cancer, which modulate receptor kinase domains that are upstream of JNK such as EGF receptor tyrosine kinase inhibitors, it is crucial to understand the off-target effects on these inhibitors on other constituents of tumor tissues including TAMs. Alterations in those upstream targets could reduce or enhance pro-tumorigenic properties of TAMs on donor-specific basis.

We also showed that although cysteine cathepsins from macrophages are important determinants of cancer cell invasion, ratio between macrophage and cystatin C also contributes toward invasion. It is not known whether there is any transcriptional or signaling feedback mechanism between cathepsins and their inhibitor cystatins. This mechanism should be investigated further as the implications for this mechanism in regards to proper dosing of the inhibitors to minimize adverse effects extend far beyond treatment of cancer. Cathepsin inhibitors have been suggested for adjuvant therapy for cancer and many other cathepsin inhibitors are in clinical trials to treat osteoporosis as well as inflammatory and autoimmune diseases. [24, 39, 45, 154, 12]

Not much is known about the signaling pathways that regulate cathepsin activity

and cystatin C levels or whether their regulation is connected. A study showed TNF-alpha increases cathepsin activity in endothelial cells via JNK pathway.[83] From the current study, we observe that inhibiting JNK decrease macrophage cathepsin activity for the donor cohort and cystatin C for selected donors suggesting a potential connection between JNK and cystatin C expression. The JNK pathway has been implicated in macrophage differentiation and polarization.[150, 68, 65] Although it is not in the scope of the current study, when expression of macrophage marker CD68 was examined using flow cytometry for donor 3 in the cohort, more than 90% percent of the macrophages treated with JNK and PI3K inhibitor expressed CD68. These cells also seemed to exhibit different macrophage subtypes between the treatment groups, suggesting that JNK inhibition may drive them toward M2-phenotype and Akt inhibition toward M1-phenotype. Studies on cysteine cathepsin expression in different macrophage subtypes is limited but can have interesting implications not only in cancer but other diseases such as atherosclerosis, where M1 and M2 macrophages either contribute to or protect against disease development and progression

Lastly, we also show that individuals with p-JNK^{low} circulating monocytes had the greatest macrophage cathepsin activity and invasion potential and these were significantly reduced upon JNK inhibition. This finding suggests that subset of patients may benefit from multifactorial screening that will include not only profiling tumor cells but also infiltrating and circulating monocytes for personalized prognosis and treatment regimen.

CHAPTER V

PROTEOLYTIC PROFILE OF M1/M2 MACROPHAGES AND INTERPATIENT VARIABILITY IN MACROPHAGE POLARIZATION

5.1 Introduction

Tumor associated macrophages (TAMs) that are differentiated from circulating monocytes promote angiogenesis,[102, 103, 104, 37, 165, 131] tumor growth[103], invasion and metastasis[109] through secretion of tissue-remodeling cathepsin proteases and cytokines to coordinate tumor-promoting immune responses.[6, 175, 120, 121] Moreover, infiltration of TAMs is often associated with poor prognosis.[34, 97, 100, 102, 22] When stimulated by distinctive cytokines, monocytes differentiate into tumor-suppressive M1 type macrophages or tumor-promoting M2 type macrophages [175], the latter being the dominant phenotype of TAMs.[175, 125] TAM/M2 type macrophages express CD206 and CD163 on their surface and secrete immunosuppressive cytokines such as IL-10[211], transforming growth factor-β (TGF-β), CCL18[91] and CCL225. Unlike their counterpart, M1 macrophages are potent tumor-suppressing cells that express CCR7 on their surface and produce iNOS, IL-1β and TNFα.[124] Functional correlations between M1/M2 phenotypes, their cathepsin protease activity profile, and the consequences this profile has in supporting or suppressing tumor growth remain to be investigated.

In atherosclerosis, monocytes differentiate and polarize into M1 or M2 macrophage subtypes in vascular walls based on the cues they have received from their environment.[140] Studies suggest that M2 (or alternatively activated) macrophages are anti-inflammatory

and anti-atherosclerotic, whereas, M1 (or classically activated) macrophages are pro-inflammatory and pro-atherosclerotic.[13, 14, 166, 126, 67] As atherosclerotic plaque development progresses, the population balance shifts from M2 macrophage-dominant to M1 macrophage-dominant[85] suggesting that the balance between M1 and M2 macrophages are important for the progression and severity of atherosclerosis.[123] Protease activity profiles of different macrophage subtypes that remodel vascular tissue also remain to be elucidated

It is not known whether there is interpatient variability in M1/M2 macrophage ratio. Also unknown is its implication on type of disease different individuals are susceptible to, or on their propensity for greater tissue remodeling and disease progression. However, based on the findings that TAMS with M2 phenotype secrete cathepsins to promote tumor growth and our finding showing interpatient variability in macrophage cathepsin activity, we test they hypothesis that M2 macrophage have greater cathepsin activity and that the patients with greater macrophage cathepsin activity have a lower M1/M2 ratio.

5.2 Materials and Methods

5.2.1 Primary monocyte isolation, differentiation and polarization

Heparinized venous blood from healthy volunteers was diluted 1:1 in sterile PBS, layered on Ficoll-Paque (GE healthcare), and centrifuged at 400g for 30 minutes. The buffy coat layer was isolated, red blood cells lysed, and peripheral blood mononuclear cells (PBMCs) were washed 3 times in PBS. Monocytes adhered overnight were cultured in RPMI containing 10% male human AB serum and 30ng/μl macrophage colony stimulating factor (M-CSF, Peprotech). Medium was replaced every 3 days. In some cases, differentiating macrophages were stimulated with IFNγ +/- LPS for M1 polarization and with IL-4 for M2 polarization in addition to M-CSF.

5.2.2 Flow cytometry

To determine M1/M2 polarization, macrophages were differentiated for 14 days, followed by harvesting and collection via centrifugation on day 15. Fc receptors were blocked using blocking buffer solution (Biolegend Human TruStain FcXTM) for 10 minutes in room temperature. Cells were then labeled with the following antibodies: PE-Cy5-CD11b, PE-CD163, APC-CD206, APC-Cy7-CCR7 (Biolegend) for 20 minutes in 4 °C in the dark. Cells were washed twice, then fixed with 4% formaldehyde for 20 minutes in the dark at room temperature. Following permeabilization using 0.02% Triton X-100, cells were stained with FITC-CD68 (Biolegend). Either isotype controls or Fluoresecnet Minus One (FMO) controls were used for fluorescence compensation. Cells were analyzed using BD LSRII, BD Aria Cell Sorter or BD Accuri and sorted using BD FACSAria (BD Biosciences).

5.2.3 Multiplex cathepsin zymography

Cell extracts from monocytes and FACS-sorted macrophages along with conditioned media from macrophages were collected. To prepare conditioned media, differentiation media was replaced with serum-free media on day 14 and incubated with the cells overnight. Conditioned media was collected and concentrated using VivaSpin®500 Centrifugal Concentrator (Vivaproducts). Cellular protein was extracted in lysis buffer (20 nM Tris-HCl at pH 7.5, 5 mM EGTA, 150 mM NaCl, 20 mM β-glycerol-phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.1% Tween-20) with 0.1 mM leupeptin freshly added. Cathepsin zymography was performed on cell extracts and on conditioned media as described previously.[202] Briefly, equal amounts of protein in non-reducing loading buffer were separated on 12.5% SDS-polyacrylamide gels containing 0.2% gelatin at 4 °C. Enzymes were renatured, and the gels were incubated overnight at 37 °C in phosphate buffer, pH 6, with 1 mM EDTA and freshly added 2mM DTT. Gels were then rinsed with dH_2O, stained with

Coomassie blue, and imaged using an ImageQuant LAS 4000 (GE Healthcare). Densitometry was performed using ImageJ to quantify the intensity of the white cleared band of proteolytic activity.

5.3 Results

5.3.1 Interpatient variability in expression of M1/M2 phenotypic markers

Freshly isolated monocytes were differentiated into macrophages with M-CSF for 14 days. Cells were collected and labeled with monocyte/macrophage markers (CD11b and CD68), a M1 macrophage marker (CD197 or CCR7) and M2 macrophage markers (CD206 or mannose receptor and CD163). The expression level was measured using flow cytometry. Although M-CSF has been shown to promote M2 polarization,[127, 73] our results show that differentiated macrophages express both M1 and M2 markers with varying degrees of expression between donors (Figure 17).

5.3.2 IL-4 stimulation increases both mannose receptor (M2 marker) and CCR7 expression level (M1 marker)

Based on our finding that there are mixed subpopulations of macrophages for individual donors, we hypothesized that monocytes have been pre-conditioned by circulating factors prior to isolation. We stimulated differentiating monocytes with IL-4 which is known to promote M2 polarization. After 14 days, cells were collected and labeled with M1/M2 surface markers, and expression levels were measured using flow cytometry. IL-4 stimulation increased expression of CD206 (M2 marker) but decreased expression of another M2 marker CD163 (Figure 18 A,B) . Surprisingly, IL-4 stimulation also increased expression of M1 polarization marker CD197 (CCR7) (Figure 18 B).

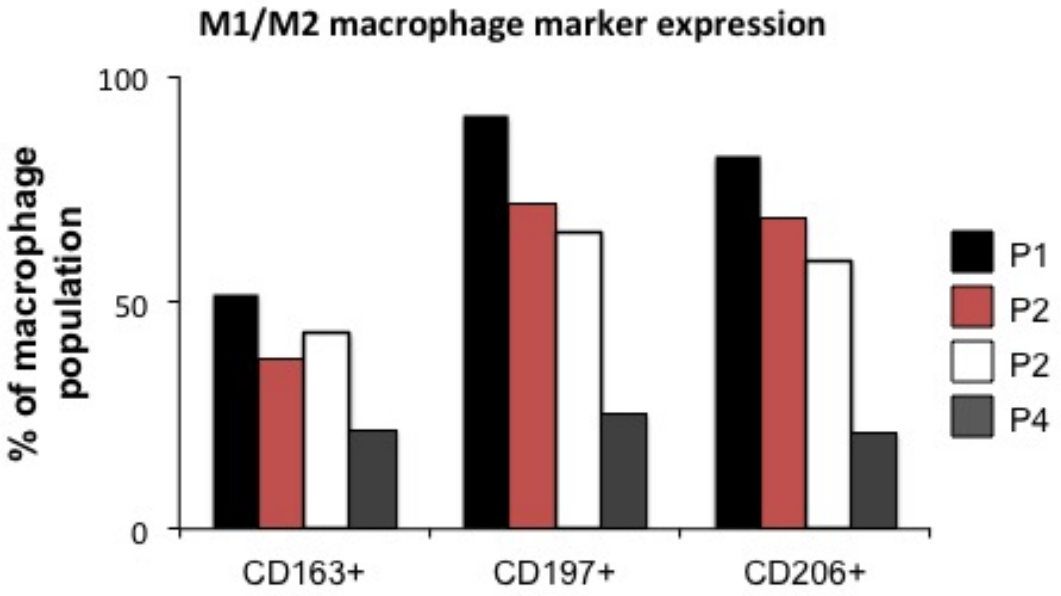

Figure 17: Interpatient variabiltiy in expression of M1/M2 macrophage markers. Freshly isolated monocytes from 4 donors were simulated with 30ng/ml M-CSF for 14 days. Expression level of macrophage polarization markers were measured using flow cytometry. Among CD11b+/CD68+ macrophages, percentage of cells expressing CD163 (M2 marker), CD197 (CCR7, M1 marker) or CD206 (mannose receptor, M2 marker) was measured and compared between donors. There were cells expressing more then one marker.

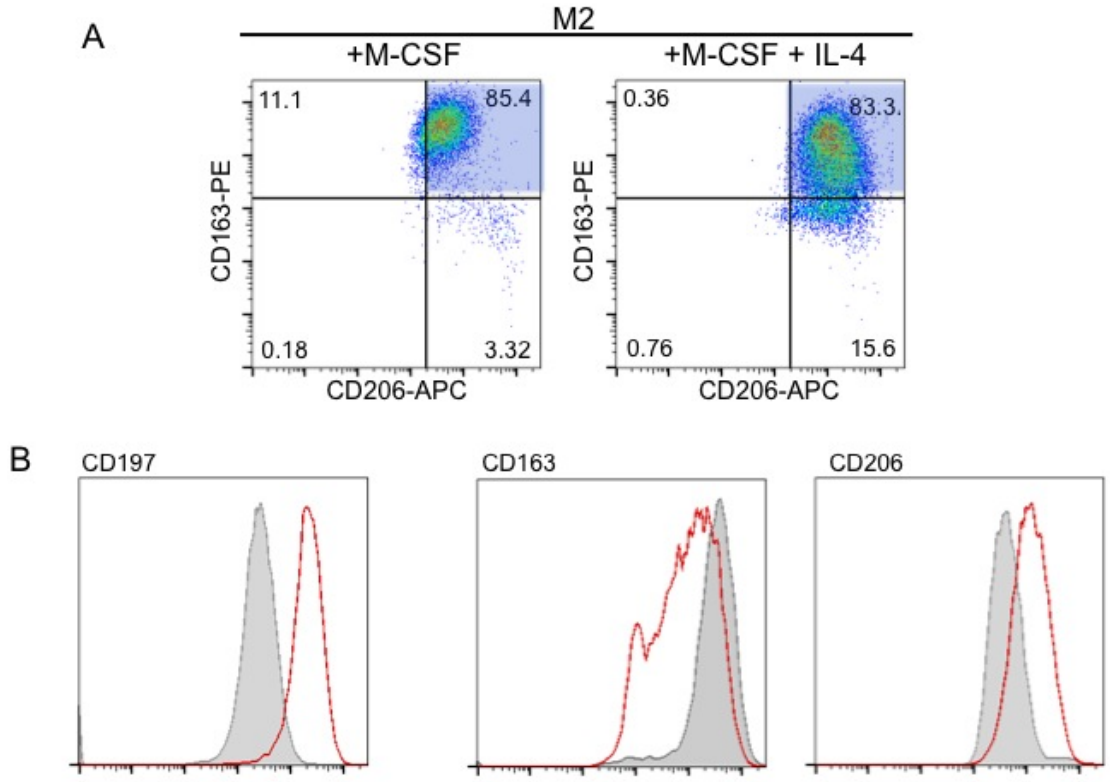

Figure 18: IL-4 stimulation increases mannose receptor and CCR7 expression level but decrease CD163 expression Freshly isolated monocytes were stimulated with both M-CSF and IL-4 for 14 days as they differentiated into macrophages. (A) Co-stimulation with M-CSF and IL-4 increased the number of cells expressing CD206, but decreased CD163 expressing cells, both of which are M2 polarization markers. (B)IL-4 stimulation also increased CD197 (CCR7) which is known to be a M1 polarization marker.

5.3.3 M2 macrophages have higher cathepsin V and S activity; M1 macrophages have higher cathepsin L activity

Although M1 macrophages are traditionally known to be pro-inflammatory and M2 macrophages anti-inflammatory, not much is known about their distinct proteolytic profile. To investigate this, freshly isolated monocytes from 3 donors were stimulated with 30ng/ml M-CSF alone, 30ng/ml M-CSF + IL-4, M-CSF + IFN-γ or M-CSF + IFN-γ + LPS for 14 days as they differentiated into macrophages. On day 15, lysates were collected and loaded for multiplex cathepsin zymography. The amount of active cathepsins was quantified with densitometry and averaged across all three patients. As a population, IL-4 stimulation increased the amount of active 35kDa, 25kDa and 20kDa cathepsins. IFN-γ stimulation increased active 25kDa cathepsin level, while IFN-γ + LPS decreased active 35kDa and 25kDa cathepsins, but increased active 20kDa cathepsin level. (Figure 19)

5.3.4 IFN-γ stimulation increases cystatin C level.

In tissues, the amount of cathepsins that are proteolytically active is determined not only by the concentration of cathepsins but also by the amount of cystatin C bound to cathepsins which inhibits cathepsin activity. Therefore, we next measured cystatin C levels from conditioned media of differentiated and polarized macrophages. After stimulating differentiating monocytes with M-CSF alone, M-CSF + IL-4, M-CSF + IFN-γ, or M-CSF + IFN-γ + LPS for 14 days; conditioned media was collected from day 14-15, concentrated, and loaded for Western blotting to measure cystatin C level. IFN-γ stimulation increased cystatin C protein expression level. (Figure 20)

5.4 Discussion

Here we showed that there is patient-to-patient variability in macrophage polarization upon stimulation with M-CSF, a differentiation and survival factor for macrophages. We also showed that stimulating differentiating monocytes with IL-4, a cytokine

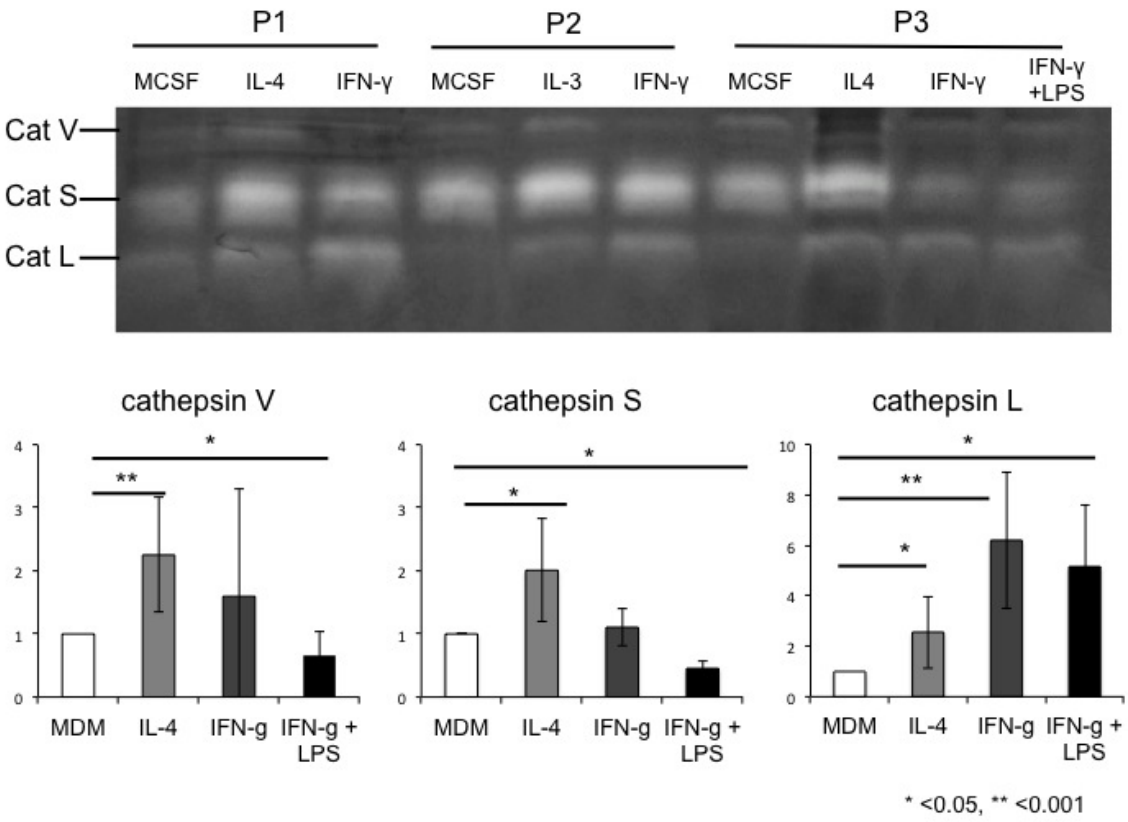

Figure 19: M2 macrophages have higher cathepsin V and S activity; M1 macrophages have higher cathepsin L activity Freshly isolated monocytes from donors were stimulated with M-CSF alone, M-CSF + IL-4 for M2 polarization, M-CSF + IFN-γ, or M-CSF + IFN-γ + LPS for M1 polarization for 14 days as they differentiated into macrophages. On day 15, lysates were collected and loaded for multiplex cathepsin zymography. The amount of active cathepsins was quantified. IL-4 stimulation increased the amount of active 35kDa, 25kDa and 20kDa cathepsins. IFN-γ stimulation increased active 25kDa cathepsin level, and IFN-γ + LPS decreased active 35kDa and 25kDa cathepsins, but increased active 20kDa cathepsin level.

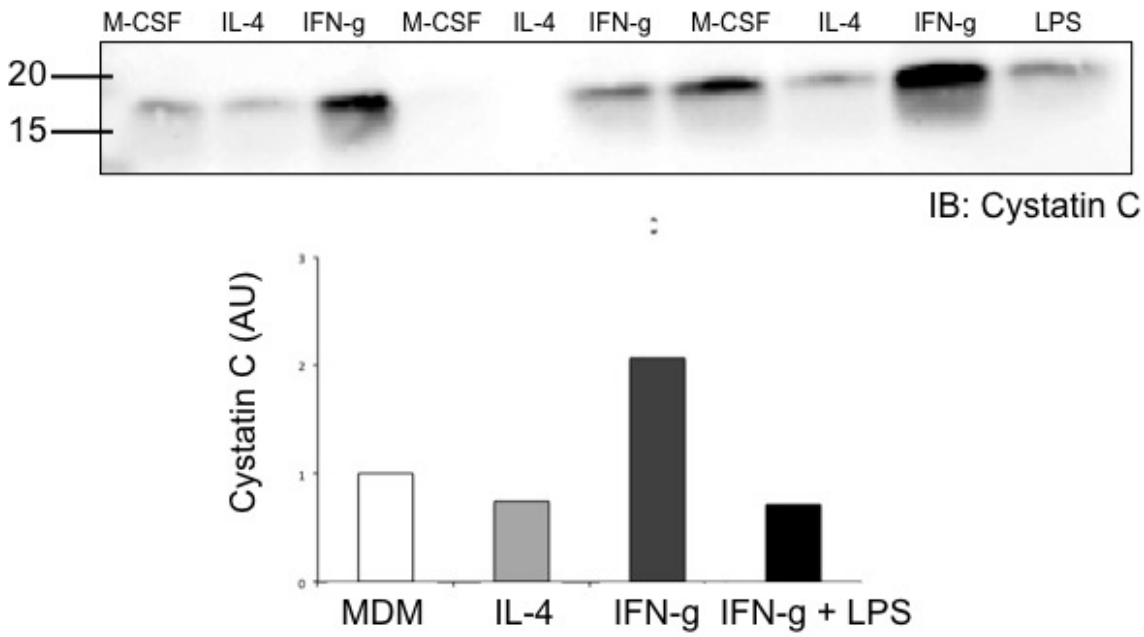

Figure 20: IFN-γ stimulation (promoting M1 polarization) increases cystatin C level Freshly isolated monocytes from donors were stimulated with M-CSF alone, M-CSF + IL-4 for M2 polarization, M-CSF + IFN-γ, or M-CSF + IFN-γ + LPS for M1 polarization for 14 days as they differentiated into macrophages. On day 15, secreted cystatin C level was quantified using Western Blotting. IFN-γ stimulation, which promotes M1 polarization, increased cystatin C level.

known to drive M2 polarization, or IFN-γ, a cytokine known to drive M1 polarization, generates mixed population of M1/M2 macrophage cells, albeit there is dominant subtype. We also observed cells that co-express traditional M1/M2 markers. This finding is in line with the shifting paradigm in macrophage polarization that rather than the M1/M2 dichotomy, macrophages exist on a continuous spectrum.[140]

Roles of different macrophage subtypes in health and disease have been studied extensively.[174, 122] However, there is a gap in the knowledge of the proteolytic profile of different macrophage subtypes. As the macrophage subtypes, as well as cysteine cathepsins, play distinct roles at different stages of tissue-remodeling diseases including cancer and atherosclerosis, it would be greatly beneficial to better understand their remodeling capacity. Findings from this study showed that M2-phenotype, driven by IL-4 stimulation, has greater cathepsin V and S activity, whereas M1-phenotype, driven by IFN-gamma, has higher cathepsin L activity. With the finding that some patients have greater expression of M1 or M2 markers, one potential mechanism underlying interpatient variability in macrophage cathepsin activity we have identified is differential make up of M1/M2 macrophages between patients. With further studies to correlate M1/M2 ratio and the net protease activity with measurable disease outcomes such as tumor invasion, it can be used as an additional prognostic markers for personalized medicine.

CHAPTER VI

VARIABILITY IN MONOCYTE CATHEPSIN ACTIVITY AMONG PATIENTS WITH BREAST CANCER

6.1 Introduction

Approximately one in eight women in the United States will develop invasive breast cancer over the course of her lifetime.[142] Despite significant advances in breast cancer therapies, patient-to-patient variability in disease progression continues to complicate clinical decisions in diagnosis and treatment.[179, 152, 27, 40, 153, 180, 205] A recent study showed that the incidence of breast cancer overdiagnosis may be as high as 30%.[11] Many more studies and commentaries followed suggesting that the incidence of overdiagnosis is prevalent only among ductal carcinoma *in situ* (DCIS), a benign form of breast tumor that has potential to become invasive cancer. Currently, there is no accurate method to predict that invasive potential, and some women decide to undergo radical mastectomy to prevent malignant progression. Until recently, many studies have focused on identifying characteristics of cancer cells themselves. However, it has become clear that tumor microenvironment, which encompasses tumor cells, stromal cells, recruited immune cells, including tumor associated macrophages (TAMs) plays crucial roles in determining behavior of cancer cells.[111, 43, 48, 28] TAMs that are differentiated from circulating monocytes promote angiogenesis,[102, 103, 104, 37, 165, 131] tumor growth[103], invasion and metastasis[109] through secretion of cytokines to coordinate tumor-promoting immune responses[6, 175, 120, 121] as well as through secretion of tissue-remodeling cathepsin proteases. Moreover, infiltration of TAMs is often associated with poor prognosis.[34, 97, 100, 102, 22]

We have shown that in healthy donors, there is donor-to-donor variability in macrophage cathepsin activity, which contributes to variability in cancer cell invasion. Moreover, we have shown that for the donors with monocytes with low JNK activation, inhibiting JNK in during monocyte-to-macrophage differentiation reduces co-cultured cancer cell invasion. With that knowledge, now we investigate patient-variability in monocyte cathepsin activity and kinase activation state for women diagnosed with various stages of breast cancer the long-term goal of this study is to find early and non-invasive markers to identify patients who may develop invasive breast cancer. The hypothesis is that interpatient variability in monocyte kinase signatures and cathepsin activity persists despite disease-related cues that modify cellular behavior.

6.2 Materials and Methods

6.2.1 Patients

Peripheral venous blood and breast tissues were obtained from female patients who are undergoing mastectomy. Patients were diagnosed with various stages of breast cancer including DCIS and invasive ductal carcinoma (IDC). Informed consent was obtained prior to surgery and is in accordance with protocol approved by Georgia Institute of Technology and Dekalb Medical Center (Decatur, GA).

6.2.2 Primary monocyte isolation

Heparinized venous blood from female patients with benign and malignant breast tumors was diluted 1:1 in sterile PBS and layered on Ficoll-Paque (GE healthcare) and centrifuged at 400g for 30 minutes. The buffy coat layer was isolated, red blood cells lysed, and peripheral blood mononuclear cells (PBMCs) were washed 3 times in PBS. $CD14^+$ and $CD16^+$ monocytes were isolated using Pan-monocyte magnetic bead isolation kit (Milteny) and lysates were collected for Bioplex kinase assay and multiplex cathepsin zymography

6.2.3 Multiplex cathepsin zymography

Cell extracts from monocytes and cell extracts and conditioned media from macrophages were collected. Non-cancerous breast tissues were homogenized using homogenizer and sonicator and lysates were collected and cleared by centrifugation. To prepare conditioned media, differentiation media was replaced with serum-free media on day 14 and incubated overnight. Conditioned media was collected and concentrated using VivaSpin®500 Centrifugal Concentrator (Vivaproducts). Cellular protein was extracted in lysis buffer (20 nM Tris-HCl at pH 7.5, 5 mM EGTA, 150 mM NaCl, 20 mM β-glycerol-phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.1% Tween-20) with 0.1 mM leupeptin freshly added. Cathepsin zymography was performed on cell extracts and on conditioned media as described previously.[202] Briefly, equal amounts of protein in non-reducing loading buffer were separated on 12.5% SDS-polyacrylamide gels containing 0.2% gelatin at 4 °C. Enzymes were renatured and then the gels were incubated overnight at 37 °C in phosphate buffer, pH 6 with 1 mM EDTA and freshly added 2mM DTT. Gels were then rinsed, stained with Coomassie blue, and imaged using an ImageQuant LAS 4000 (GE Healthcare). Densitometry was performed using ImageJ to quantify the intensity of the white cleared band of proteolytic activity.

6.2.4 Detection of CD68

To measure tissue expression level of CD68, non-cancerous breast tissue lysates were loaded for Western blotting. Anti-CD68 antibody (Biolegend, 1:500 dilution) was used. Signals were detected using LI-COR Odyssey.

6.2.5 Kinase phosphorylation analysis

Freshly isolated monocytes were lysed and total protein was determined using BCA kit (Pierce). Bioplex® bead kits (BioRad) were used according to manufacturers instructions with 5 μg protein from each sample and measured phosphorylation of

ERK1/2 (Thr^{202}/Tyr^{204}, Thr^{185}/Tyr^{187}), Akt (Ser^{473}), p38 MAPK (Thr^{180}/Tyr^{182}), JNK (Thr^{183}/Tyr^{185}), c-jun (Ser^{63}), NFκB p65 (Ser^{536}) and IκB-α (Ser^{32}/Ser^{36}). Signal values for each phosphorylated kinase were normalized to the signal detected in a master lysate prepared in bulk from pre-stimulated cells that was used as a control for all assays. Signal values for each kinase were normalized to maximum values for each kinase.

6.3 Results

6.3.1 Interpatient variability in kinase activation signatures and cathepsin activity of circulating monocytes from breast cancer patients

To test the hypothesis that disease conditions do not obliterate patient variability in kinase signatures and proteolytic activity of monocytes, we isolated circulating PBMCs from female patients diagnosed with various stages of breast cancer. Whole PBMCs or monocytes were isolated using magnetic activated cell sorting (MACS) (Milteny) and lysed for cathepsin zymography and Bioplex kinase assays. (Figure 21A, B) For the first patient cohort, individual active cathepsins were quantified separately (35 kDa, 25 kDa and 20 kDa). For the second cohort, the amount of total active cathepsins was quantified (Figure 21C, D). There is clear patient-to-patient variability in monocyte cathepsin activity within the patient population. In particular, zymographic analysis of patient 4 shows uniquely high amounts of cathepsin activity at 37kDa band, which we have identified to be cathepsin K. As we have seen in healthy donor populations, there are patients who has $JNK^{low} monocytes$ (P1, 6 and 7), which may have higher cathepsin activity and invasion potential upon differentiating into macrophages.

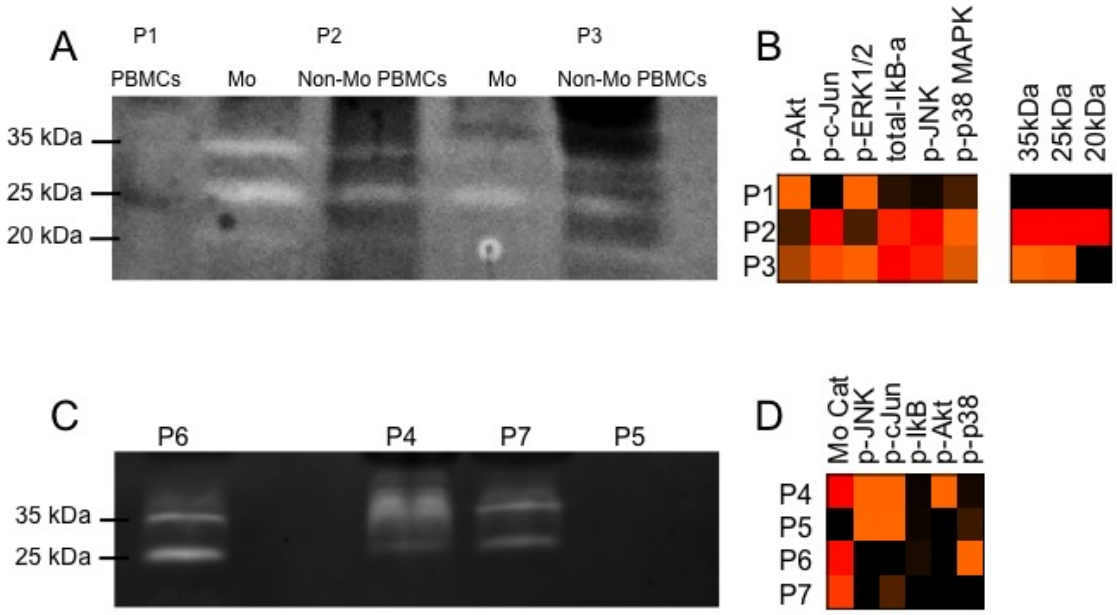

Figure 21: Interpatient variability in monocyte cathepsin activity and kinase activation signatures among patients with breast cancer. Circulating PBMCs were isolated from female patients diagnosed with various stages of breast cancer. Whole PBMCs or monocytes were isolated using magnetic beads isolation and lysed for cathepsin zymography and Bioplex kinase assays. (A,B) Zymograph and phosphorylated kinase measurements from a first cohort of patients are shown. Active among of individual cathepsins (35 kDa, 25 kDa and 20 kDa) are quantified separately. (C,D) Zymograph and phosphorylated kinase measurements from the second cohort of patients are shown. The amount of total active cathepsins was quantified.

6.3.2 Interpatient variability in cathepsin activity and number of macrophages in non-cancerous breast tissues from female patients with breast cancer

Next we tested the hypothesis that when the circulating monocytes enter tissues, whether in healthy or disease states, the patient-specific proteolytic profile of monocyte-derived macrophages is maintained. Non-cancerous breast tissues from the first cohort were obtained following mastectomy. Then the tissues were homogenized, lysates were collected and loaded for multiplex cathepsin zymography and Western blotting to detect macrophage CD68 expression levels. Patient 2 whose monocytes had the greatest cathepsin activity had the lowest cathepsin activity from breast tissue lysates (Figure 22A). Patient 1 with undetectable monocyte cathepsin activity was shown to have highest cathepsin activity and macrophage concentration in the tissue. (Figure 22B)

6.4 Discussion

In this study, we have shown that disease conditions do not eliminate patient-to-patient variability in monocyte kinase activation or cathepsin activity. There were also patients whose monocytes had low level of JNK activation, which in healthy cohort was shown to have greater cathepsin activity and invasion potential when differentiated into macrophages (Figure 16). Also JNK inhibition most effectively reduced macrophage cathepsin activity and cancer cell invasion for these healthy donors as well (Figures 13 and 14). Although it still remains to be investigated whether individual patients pre-disease monocyte kinase signature and cathepsin activity profile is conserved through development and progression of breast cancer, findings from these patients introduces the exciting possibility of reducing invasiveness for patients with the most aggressive tumors. Furthermore, monocyte kinase and cathepsin profiling would serve as a minimally invasive method to perform at patients initial visit that can inform chemotherapy regimens, as well as invasive potential of non-malignant

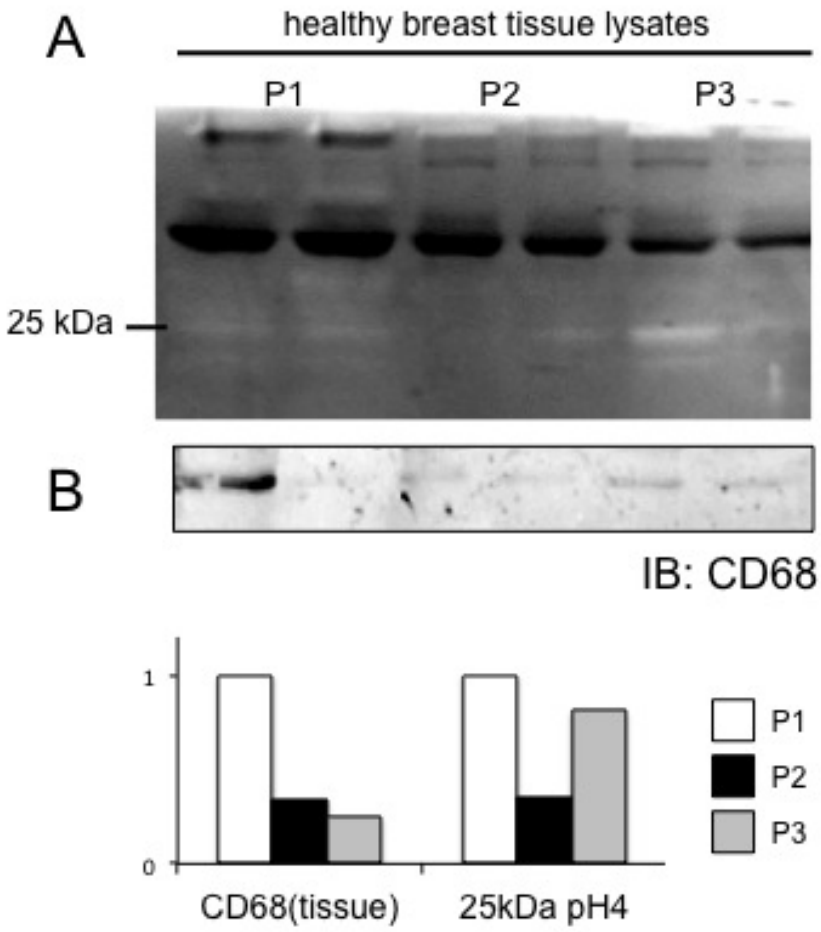

Figure 22: Interpatient variability in cathepsin activity and number of macrophages in non-cancerous breast tissues from female patients with breast cancer After patients underwent mastectomy, non-cancerous part of breast tissue was collected, homogenized and loaded for multiplex cathepsin zymography to measure the amount of active cathepsins (A) and Western blotting to quantify expression of macrophage marker CD68 (B).

DCIS, which has the highest incidence of overdiganosis.

It has long been suggested that chronic inflammatory states can lead to development of cancer.[6] Tumor microenvironment is comprised of myriad of immune cells and immune modulators that supports or fights cancer development.[28, 66, 173] Furthermore, recent studies have identified circulating factors or circulating tumor cells that is upregulated at different stages of cancer and can be used as a biomarker.[30] All these cues could potentially interact with circulating monocytes and the monocytes that enter tumor tissue and can affect their phenotype and proteolytic activity. Taken together, it remains to be investigated whether disease state leads to a distinct shift in kinase signatures and if so, whether the distinct individual kinase signature is still maintained despite this shift.

The results from the previous chapter suggested that individual donors could have intrinsically different M1/M2 macrophage ratio and that macrophage subtypes may have distinct cathepsin activity profile. In some cancers, M2 macrophages, or tumor-associated macrophages have been shown to promote tumor progression and are associated with poor prognosis.[95, 130] Distinct cytokines that are present in tumor microenvironment drives macrophage polarization. Therefore it should be investigated whether monocyte kinase signatures and cathepsin activity profile is maintained once they differentiate into macrophages in breast tissues, both in healthy conditions and within tumor tissues as well as whether there is predictable correlation between them. In addition, it is also unknown whether this intrinsic macrophage subtype ratio is maintained in tumor microenvironment and whether they are predictive of disease trajectory, and whether different stages of breast cancer have distinguishing monocyte kinase signature and proteolytic profile.

This study also provides a basis for in vivo studies to determine the efficacy of targeted inhibition of JNK activation in monocytes with the long-term goal of reducing invasion of the most invasive tumors. As JNK inhibition has been shown

to decrease breast cancer cell proliferation and promote apoptosis,[137] there may be a synergistic benefit in targeting the pathway. Through patient-specific analysis of monocyte kinase signature and cathepsin activity, it maybe possible to identify the right candidates for this adjuvant therapy for personalized cancer therapy.

CHAPTER VII

DISCUSSIONS

As a whole, this work was aimed to address the issues of patient variability in disease contributing phenotypes by monocyte-derived cells with special emphasis on cysteine cathepsin which mediated tissue destructive diseases namely, breast cancer. Many studies have investigated patient variability at the genomic level, with the goal of achieving personalized predictive medicine. These studies revealed helpful insights, but there are many regulatory steps between information coded in genes down to where cells are executing various functions with number of factors such as cytokines, kinases and proteases. The main contribution of the current work to the field of personalized medicine is showing that patient-variability in disease progression can be influenced by variability in cellular proteolytic activity, which can be predicted by multivariate analysis of set of kinases. As discussed previously, there are bevy of circulating factors, such as cytokines and hormones that modulates monocyte behavior but cannot be measured explicitly. However, by using a systems biology approach to link cell differentiation cues and responses through the integration of signals at the kinase level, where integration of ubiquitous information is processed intelligently by the differentiating cell, we were able to mathematically predict relative amounts of cathepsin activity and distinguish the donors with higher cathepsin activity. Implication of this finding in furthering personalized medicine is that with the analysis of kinase signatures of monocytes isolated through a simple blood draw from individuals, patient-specific disease potential could be predicted without extensive profiling of cytokines and other circulating factors.

Cathepsin activity has been mechanistically linked to malignant transformation,

angiogenesis, tumor invasion, growth, metastasis in context of tumor,[138, 55, 81, 207, 178, 21, 90, 203, 62, 197, 172, 143, 110, 57, 200] to transmigration of monocytes, macrophage lipid metabolism, elastic lamina degradation and plaque rupture in the context of atherosclerosis.[157, 158, 156, 185, 117, 26] Because of this, tissue levels of cathepsins are not just surrogates, but functional biomarkers whose level could be directly correlated to disease progression and inform treatment strategy. Cathepsin B, in particular, perhaps due to its abundance in cells, has been investigated as biomarker for cancer over 30 years.[176] Cysteine cathepsins K, L, S, V and X have been investigated as well with mixed success.[10, 46] However, it has been difficult to reliably measure the amount of individual active cathepsins, as cathepsins are very closely related structurally. In addition, the ubiquitous presence of the endogenous inhibitors, cystatins and stefins can interfere with accurate measurement of active cathepsins. There is also a lack of a consensus to report cathepsin gene expression, pre-, mature-, or total cathepsin protein level or activity level as biomarkers. The later obstacle could be attributed to substrate cross reactivity and closely related enzyme structure. Much effort has also been made to inhibit cathepsin activities for therapeutic values.[154, 193, 151, 198] The most heavily targeted cysteine cathepsin is cathepsin K for its role in bone resorption that occurs during osteoporosis and bone metastasis.[99, 18] However, only the cathepsin K inhibitor, odanacatib successfully completed phase III clinical trial.[182] Most clinical trials were halted due to side effects or lack of therapeutic efficacy. Both biomarker studies and drug trials investigate the patient cohort as a one large unit instead of examining individual differences. This approach is necessary of course to elucidate disease-specific mechanisms, biomarkers or treatment efficacy with statistical power. However, interpatient variability can reduce statistical power and can negate patient-specific effects. For example, it was shown in the current study that on a population level, there seemed to be no significant effect on cystatin C level and cancer cell invasion upon inhibiting JNK in

differentiating monocytes. However, when examined closely at the individual patient level, there were patients who indeed responded to the inhibition with decrease or increase in these outcomes. Although identifying patient-specific biomarkers and responses to therapy can be time consuming and cost-prohibitive, personalized approach is becoming much more feasible and potentially more efficient and effective with the rapid advancement in computational biology and omic-scale analysis,[147, 54, 195] This work suggests that in addition to genomic and metabolomics analysis, personalized medicine could benefit from systems-scale analysis of kinases and proteolytic networks.

Furthermore, studies have shown that macrophages and cathepsins can blunt the effect of chemotherapeutic agents.[172, 32, 5] Although, there are many tumor-specific and patient-specific factors that confers resistance to chemotherapies,[35, 210] results from this study suggest a potential new mechanism of resistance for certain patients. Specifically, patients with higher macrophage cathepsin activity may have greater potential for developing resistance. However, the finding that among these patients, JNK inhibition effectively reduces macrophage cathepsin activity can be used to improve treatment efficacy of main chemotherapeutics through reduced resistance.

Results from this work also suggested donor-to-donor variability in the makeup of polarized macrophage subtypes. Although traditionally, macrophage polarization was known to be a dichotomy, now studies have shown there is a continuous spectrum of macrophage phenotypes and they can switch between the subtypes based on environmental cues.[174, 140] For example, in atheroma, a heterogeneous population of macrophage subtypes are found, where the M1-phenotype promotes inflammation and atherogenesis and M2-phenotype is atheroprotective.[123] In the current work, when donor monocytes were stimulated with a single cue, M-CSF, resultant macrophage population displayed mixed M1 and M2 phenotypic markers. We can hypothesize

that patient-specific circulating cues have primed monocytes prior to isolation, driving preferential polarization. These phenomena may lead to a greater propensity for inflammatory diseases or provide greater protection against them. Further investigation on this interpatient variability could reveal additional predictive metric for patient-specific disease potential.

Disease state introduces an altered or new set of cues that can modulate proteolytic behavior of circulating monocytes and differentiating macrophages that have entered tumor tissue or developing plaques. Questions have been raised whether disease conditions equalize or reduce patient-to-patient variability by preconditioning circulating cells with similar disease-specific cues. Results from this work suggest that the patient-variability is maintained. Further work with a larger patient cohort along with the comparison to a healthy population will be able to more clearly elucidate the effect of disease state on patient-variability. However, we were able to identify patients with monocytes with low JNK activation. In the healthy population, monocytes from these individuals when differentiated into macrophages had high cathepsin activity and invasive potential, which were significantly reduced upon JNK inhibition. Therefore it is promising that these metrics can be used to identify breast cancer patients who may be develop more aggressive cancer and can benefit from targeted JNK inhibition.

Findings from the current study could be applied to other tissue-destructive diseases mediated by cathepsins and monocyte-derived cells such as macrophages. Atherosclerosis is another tissue remodeling diseases where cathepsins and macrophages have been shown to play key roles in disease development and progression.[31, 88, 115, 114, 116, 185, 184, 24, 186, 117] The earliest changes that leads to atherosclerosis occurs in endothelial layers at sites of low, and oscillatory shear stress such as arches and bifurcations. Cathepsin expression and activity is regulated by shear

stress and low and oscillatory shear stress have been shown to upregulate expression and activity of cathepsins K and L by endothelial cells.[157, 158, 156] Cathepsin S was shown to aid macrophage transmigration through endothelial monolayer in vitro.[185] Cathepsins also play roles in lipid metabolism, which furthers atherosclerotic plaque formation.[117] In turn, increased levels of oxidized LDL, which leads to foam cell formation, was shown to disrupt lysosomal membrane, leading to relocation of cathepsins B and L to cytoplasm.[26] As atherosclerosis progresses, cathepsins secreted by macrophages degrade elastic lamina and facilitate migration of smooth muscle cells, which produces collagen as well as cathepsin K, into neointimal space. Higher plaque collagen content is correlated with increased plaque stability. Furthermore, the pathophysiological importance of cathepsins in atherosclerosis has been demonstrated in double-knockout mice deficient in apolipoprotein E (ApoE) and cathepsins K, S or L, which showed a reduction in the number and size of atherosclerotic lesions, decreased fragmentation of the elastic lamina, and decreased plaque rupture.[116, 184, 164, 88] Double-knockout mice deficient in ApoE and Cystatin C experienced an increase in atherosclerosis.[9] Even osteoclasts have been implicated in later stages of atherosclerotic plaque calcification and shown to be differentiated from infiltrated monocytes.[2, 1, 133] Therefore, findings from this study has an implication that those individuals with greater cellular cathepsin activities may have greater propensity of developing atherosclerotic plaques or greater risk of plaque rupture and merits further investigation for personalized medicine strategies.

Patient-to-patient variability seen in these diseases has been an added challenge for physicians to properly diagnose and treat their patients. This work demonstrates that interpatient variability at the level of cellular protease activity and other disease-mediating phenotypes must be examined in addition to genomic analysis. Incorporating this knowledge for patient-specific diagnosis and treatment can bring physicians one-step closer to the ultimate goal of personalized medicine.

CHAPTER VIII

CONCLUSIONS

Cathepsins have long been investigated as biomarkers and therapeutic targets in cancer and cardiovascular diseases. Patient-to-patient variability seen in these diseases has been an added challenge for physicians to properly diagnose and treat their patients. This work illustrates the importance of investigating interpatient variability not only at the genomic level, but also at the level of cellular protease activity and other phenotypes that mediate disease progression. Incorporating this knowledge for patient-specific diagnosis and treatment can bring physicians one-step closer to the ultimate goal of personalized medicine.

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